THE MURINE CELL-MEDIATED IMMUNE RESPONSE TO ADENOVIRUS RECOMBINANT AdG12

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ABSTRACT

This study was undertaken to examine the specificity of the cell-mediated immune response to vesicular stomatitis virus in mice, using the recombinant adenovirus vector AdG12. AdG12 contains the coding region for VSV glycoprotein (G) within the genome of adenovirus type 5. Ultimately, these studies attempted to provide a model for the use of adenovirus vectors to elicit specific CTL responses in mice against an inserted foreign protein.

Cell-mediated immunity was examined using a standard ^{51}Cr release assay. Splenocyte effectors from VSV or AdG12 primed mice were tested for their ability to lyse labelled infected target cells.

A number of target cell lines were analyzed for productive infection by AdG12 and expression of VSV-G. Of the lines tested, B10.D2 ($H\text{-}2^d$) and PAK ($H\text{-}2^b$) lines were shown to be infectible with AdG12 and expressed VSV-G 36 hours post infection. Cell lines P815 ($H\text{-}2^d$) and EL-4 ($H\text{-}2^b$) did not appear to be AdG12 infectible.

Responses were measured in mice intravenously infected with AdG12. Results demonstrated that peak cytotoxic activity from AdG12 primed mice occurred six days post-

infection against syngeneic target cells infected with AdG12, Ad5 wt or VSV. However, these effectors also significantly lysed allotargets infected with VSV, implying that VSV infected targets were lysed in a non-MHC restricted manner.

In subsequent experiments, it was discovered that VSV infected B10.D2 and PAK targets were markedly lysed by effectors from immunized and non-immunized Balb/c, C57B1/6 and CBA/J mice. Thus, it appeared that these mouse strains contained an inherent or natural cytotoxic activity against VSV infected targets that was unlike classical CTL killing.

Depletion experiments showed that this activity was not due to adherent or Thy1 bearing cells within spleen cell populations. To further characterize this activity, splenocyte effectors were tested for their ability to lyse NK-sensitive YAC-1 targets, but no significant lysis was demonstrated. However, despite these results, it appeared that this activity was that of an NK-like effector.

The presence of NK-like cytotoxicity against VSV infected targets precluded efforts to define specific anti-VSV responses in these mice.

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LIST OF ABBREVIATIONS

Ad	adenovirus
APC	antigen presenting cell
CPE	cytopathic effects
CTL	cytotoxic thymus derived lymphocyte
E3	early coding region 3 of adenovirus
EM	Electron microscopy
G	internal glycoprotein of VSV
HA	virus-encoded hemagglutinin protein
HSV	herpes simplex virus
IFN	interferon
IL-2	interleukin-2
L	RNA polymerase protein of VSV
LCMV	lymphocytic choriomeningitis virus
LT	lymphotoxin
M	matrix protein of VSV
MCMV	murine cytomegalovirus
MHC	major histocompatibility complex
MHV	murine hepatitis virus
MS	Mudd-Summers strain of VSV Indiana
N	nucleocapsid protein of VSV
NK	natural killer cells

NP	virus-encoded nucleoprotein
NS	nonstructural protein of VSV
OVA	ovalbumin
PEC	peritoneal exudate cells
PFP	pore forming protein; Perforin; Cytolysin
polyA	poly-Adenylation
SJ	San Juan strain of VSV Indiana
TcR	T-cell receptor for antigen + MHC
TK	Thymidine kinase
ts	temperature sensitive mutant virus strain
UV	ultraviolet light; to inactivate virus
VSV	vesicular stomatitis virus

A/. INTRODUCTION

Vesicular stomatitis virus (VSV) belongs to the family Rhabdoviridae or Rhabdoviruses. The term rhabdovirus is derived from the greek "rhabdos" meaning rod and refers specifically to the shape of these viruses (although they are in fact bullet shaped or bacilliform) (Brown, 1987). First used in 1966 by Melnick and McCoombs, the term was later recommended by the International Committee on Nomenclature of Viruses (Wildy, 1971). Rhabdoviruses are all bullet shaped and infect a number of vertebrate and invertebrate host organisms as well as plants and protists. Thus they are subdivided into several genera. The genus Vesiculovirus represents nine virus types, so named because they generally cause vesicular lesions in the mouth, teats and coronary band of the foot of infected hosts (Hanson, 1970). This genus includes the two serotypes of VSV. One serotype, Indiana, is divided into subtypes Indiana, Cocal, Argentina, and Brazil. The other serotype, New Jersey includes Concan and Hazelhurst subtypes.

The first studies on VSV were done by Chow et al. (1954). VSV was of particular interest because it caused a distinct disease in cattle similar to Foot and Mouth disease, and several outbreaks of the Vesicular Stomatitis disease had been reported since 1901 (Hanson, 1952). Bradish et al. (1956) isolated VSV from disease lesions and used electron microscopy (EM)to discover its bullet shaped morphology.

Further analysis showed that VSV could be grown and purified with ease in a variety of tissue culture systems. In this respect, much information regarding the morphology and pathogenesis of VSV has been obtained.

A/1. MORPHOLOGY OF VSV

As mentioned, the infectious (standard) virion of VSV is bullet shaped i.e. round at one end and flat at the other, although Orenstein et al. (1976) claimed that this shape was an artifact of fixation and staining and that VSV was actually bacilliform (rounded at both ends). Depending on the strain, VSV has an approximate length of 180 nm and a width of 65 nm (Wagner, 1975). Along with this standard particle, there are truncated (T) or defective interfering (DI) particles that are commonly found in uncloned preparations of VSV. These are approximately the same width as standard particles but vary in length from 50 to 80 nm depending on the amount of viral RNA deleted (Huang and Baltimore, 1977).

By EM analyses, VSV has been shown to consist of a tightly coiled nucleocapsid surrounded by a membrane or envelope. Within this envelope, VSV and other rhabdoviruses have protruding spikes about 10 nm long, readily removed by exposure to proteases (Wagner, 1987). Overall, VSV is composed of approximately 74% protein, 20% lipid, 3% carbohydrate and 3% RNA (McSharry and Wagner, 1971). Lipid makes up the external envelope, which is a classic lipoprotein

bilayer. The VSV membrane is about 50% lipid and 50% protein. The lipids are derived entirely from the host cell but are selected in different proportions than the host cell plasma membrane. For example, VSV lipid contains more cholesterol and there are greater amounts of amino-phospholipids (McSharry and Wagner, 1971). This altered lipid composition contributes to a greater viscosity of the VSV membrane compared to that of the host cell membrane from which it was derived (Barenholz et al., 1976). VSV membranes contain two viral protein species: an externally oriented integral glycoprotein (G) and a peripheral matrix (M) protein that lines the membranes' inner surface (Patzer et al., 1979). G protein is the major antigenic determinant responsible for VSV type-specificity and gives rise to neutralizing antibodies in an infected host (Kelley et al., 1972; Volk et al., 1982). M protein acts as a "glue" to attach internal nucleocapsid to a cell membrane upon budding of the virion. M protein is very basic (pI ≈ 9.1) and inhibits transcription by binding to the nucleocapsid (Carroll and Wagner, 1979).

If the envelope is disrupted using detergent, the internal nucleocapsid is released. This nucleocapsid retains a secondary tightly coiled structure in a salt free solution, but uncoils in solutions of high ionic strength (Newcomb and Brown, 1981). A number of studies have determined the components of the uncoiled nucleocapsid. It is in fact an association of viral genome with three particular proteins

(Schubert et al., 1984). The secondary structure is due to electrostatically bound M protein which dissociates from the VSV nucleocapsid in hypertonic solutions (Emerson and Wagner, 1972; Newcomb and Brown, 1981). The VSV genome is an unsegmented single strand of RNA that is complimentary to messenger RNA and is therefore referred to as negative stranded. This RNA genome alone is not infectious (Huang and Wagner, 1966). Infectious RNA is associated with a major nucleocapsid (N) protein, tightly complexed with virion RNA, dissociating only with SDS (Gallione et al., 1981). In fact VSV nucleoprotein (RNA-N) is resistant to ribonuclease digestion even after exposure to high salt (Emerson and Wagner, 1973). Infectious nucleocapsid also contains two other minor proteins, the larger L protein and the nonstructural phosphorylated NS protein (Bishop and Roy, 1972). L and NS are associated with the nucleocapsid template to form an endogenous RNA-dependant RNA polymerase (Emerson and Yu, 1975). Both can be dissociated from the nucleocapsid under varying conditions of high ionic strength, resulting in the loss of transcriptase activity and virus infectivity (Emerson and Wagner, 1973). The single stranded VSV genome has been analysed extensively using cDNA cloning. It has a highly conserved size and a sequence composed of 11,161 nucleotides (Wagner, 1975). Transcription of the genome proceeds left to right, generating a short untranslated leader RNA of 47 nucleotides followed by monocistronic mRNA's that

correspond to five viral proteins (Rose and Schubert, 1987). Replication occurs via a full length positive stranded copy of the genome. Both expression and replication are strategically balanced due to a particular organization of the genome such that the five genes are in a specific conserved order. Studies by Abraham and Banerjee (1976) and Ball and White (1976) deduced the transcriptional and translational map of the VSV genome by UV irradiation. Increasing exposure of VSV particles to UV light caused differential inhibition of synthesis in vitro of individual mRNA species coding for each viral protein (Abraham and Banerjee, 1976). By analysing the synthesis kinetics of each mRNA species and comparing message size to the size of the corresponding gene, they found that each mRNA species is synthesized sequentially in the order 3'-N-NS-M-G-L-5'. RNA duplex mapping using genomic RNA and isolated mRNAs confirmed these results and established the complete primary structure of the VSV genome (Herman et al., 1978). Once transcription is initiated at the 3' end of the genome, there is a process of attenuation at or near each gene junction resulting in a decrease in the amounts of message synthesized depending on the distance of each corresponding gene from the 3' terminal start of transcription (Iverson and Rose, 1981). For example, the polymerase gene L is positioned furthest away from the transcription start site and as such is transcribed in low amounts compared to the N gene. N is in fact needed in large

amounts for encapsidation of genomic RNAs, where L is only needed in catalytic amounts (Villareal et al., 1976). Therefore, the organization of the genome along with a sequential and attenuated mode of transcription ensures that each gene product is synthesized in ratios needed throughout infection.

A/1.1. The Proteins of VSV

VSV contains a single glycoprotein (G) forming spikes on the virion surface. There are approximately 1200 molecules of VSV-G per virus particle, and these are thought to be in monomeric form (Thomas et al., 1985). Antibodies that neutralize virus infectivity are directed against G protein (Kelley et al., 1972). There is a 511 amino acid precursor form of G protein that includes a 16 amino acid signal sequence (Irving et al., 1979, Rose and Gallione, 1981). Also, attached to amino acids 179 and 336 are two asparagine linked complex oligosaccharides (Rose and Gallione, 1981), and the fatty acid palmitate is esterified to cysteine 489 in the cytoplasmic domain of G (Rose et al., 1984). VSV-G is positioned in the virion so that the NH_2 terminal 95% of the polypeptide chain is external to the bilayer (Rose et al., 1980). This protein has two roles in the virus life cycle. Firstly, it binds virus to its host cell, inducing uptake of virus via fusion with an endosomal membrane; and, during virus maturation, there is an interaction between the internal

components of the virus and the G protein cytoplasmic domain (White et al., 1983). As reviewed by Hubbard and Ivatt (1981), VSV-G has been used as a major model for the study of transport and processing of proteins that are directed to the plasma membrane. The G protein precursor has a short hydrophobic NH_2-terminal signal sequence that initiates insertion of the nascent chain into the rough endoplasmic reticulum (rER) and is cleaved thereafter (Rothman and Lodish, 1977). Once transported through the ER and golgi apparatus, G becomes anchored stably in the membrane by a hydrophobic 20 amino acid domain that spans the bilayer (Rose et al., 1980). Without this domain, G protein is secreted. VSV infected cells release a truncated soluble form of the glycoprotein (Gs), which lacks amino acids from the COOH terminal anchor region due to premature translation termination (Little and Huang, 1978). Gs is thought to play a role in viral pathogenesis (Little and Huang, 1977). The remaining COOH terminal portion (29 amino acids) forms the cytoplasmic domain of the molecule, including cysteine 489 which is, as mentioned, palmitated (Rose et al., 1984). This fatty acid aids in the transport of G to the cell surface. The importance of VSV-G protein glycosylation was shown in studies where VSV infected cells were treated with tunicamycin (Leavitt et al., 1977; Morrison et al., 1978). This treatment inhibits N-linked glycosylation of G, resulting in failure of nonglycosylated G to reach the cell surface and complete

inhibition of virion production. If only one or the other of two glycosylation sites is deleted, using oligonucleotide directed mutagenesis, VSV-G is still transported (Machamer et al., 1985). Thus, glycosylation at only one site promotes a certain conformational change of the protein that is required for transport. The fusion activity of G protein plays a major role in VSV infection. It is only through fusion of the viral envelope with components of host cellular membrane that nucleocapsid is released from the virion into the cell cytoplasm, thus causing infection (White et al., 1983). Evidence for this activity comes from animal cells transformed to express G protein, using cDNA clones of the G gene (Florkiewicz and Rose, 1984; Riedel et al., 1984). These cells expressing G fuse together if grown in low pH medium.

The matrix (M) protein is the major structural protein of VSV virions. With 1800 molecules per virus particle, M is the most abundant of the viral proteins (Thomas et al., 1985). It has a molecular weight of 26,064, and its NH_2 terminal domain is very basic (Rose and Gallione, 1981). VSV-M is thought to play a critical role in virus assembly because mutations in M block the appearance of virus particles (Knipe et al., 1977). M protein is located inside the viral envelope and crosslinking experiments suggest that it may form a bridge between the cytoplasmic domain of G and the N (nucleocapsid) protein (Zakowski and Wagner, 1980). However, since M protein was shown to bind to the membranes of uninfected cells (Cohen

et al., 1971), Zakowski et al. (1981) suggested that M interacts with membrane phospholipids and thus allows the nucleocapsid to bind to the membrane. M protein also negatively regulates viral RNA synthesis (Carroll and Wagner, 1979). The basic NH_2 terminus of M is thought to interact with genomic RNA in this role. Others have suggested that because of its high affinity for nucleocapsid, M protein is necessary for holding it in a compact secondary structure (Newcomb and Brown, 1981).

The nucleocapsid (N) protein, as evident by its name, is associated with the VSV genome. There are about 1300 molecules of N per virus particle (Thomas et al., 1985). Both positive and negative sense leader RNAs in infected cells are found encapsidated with N within the first 14 5' terminal nucleotides (Blumberg et al., 1983). Encapsidation by solubilized N protein proceeds unidirectionally from the 5' to the 3' end of leader RNA. N associated with the RNA transcripts prevent polymerase from recognizing termination sites on the genome, resulting in synthesis and encapsidation of full length plus and minus strand genomes. Thus N seems to regulate the balance between viral transcription and replication in the infected cell (Blumberg et al., 1984). Immediately after virus entry, transcription is favoured over replication because there is no pool of N protein; later, when N is in excess, replication would be favoured. Animal cells have also been transformed to express N protein (Sprague et

al., 1983). The protein, examined by indirect immunofluorescence, is localised to the cytoplasm and has a granular appearance identical to that of N protein observed in VSV infected cells. Therefore N is thought to spontaneously aggregate and form nucleocapsid-like structures (appearing granular) in the cytoplasm (Sprague et al., 1983).

The NS protein of VSV accumulates in the cytoplasm and is the most abundant soluble protein. It runs on SDS-PAGE at 40-60 Kd, depending on the bis/acrylamide ratio of the gel (Bell et al., 1984). NS protein along with L and an N-RNA template are the vital components of VSV transcription (Emerson and Yu, 1975). The abundance of cytoplasmic NS suggests that it may have additional functions other than a role in transcription. In fact, complexes of N and NS proteins have been detected in the cytoplasm of infected cells (Bell et al., 1984). This association is thought to prevent self aggregation of N proteins and thus block nucleocapsid assembly (Arnheiter et al., 1985). There are ten different phosphorylated subspecies of NS (Hsu et al., 1982) the sites of phosphorylation being in the NH_2 terminal half of the protein (Bell and Prevec, 1985). It is not however known which of these phosphorylated forms preferentially binds to the nucleocapsid or to the L protein in regulating the process of transcription.

The largest gene in the VSV genome encodes an RNA-dependant RNA polymerase known as the L protein. Emerson and

Yu (1975) showed that both the L and the NS proteins are required for polymerase activity, where encapsidated RNA serves as the template. Only because of its exceptional size of 241 Kd (Shubert et al., 1984) and its presence in catalytic amounts of about 50 molecules per virus particle (where there are 470 NS molecules) is L suspected to be the active component for polymerase activity (Thomas et al., 1985). Since L protein binding to the template requires the presence of NS, it is suggested that L is the transcriptase and NS serves as a regulator of transcription (Mellon and Emerson, 1978). Studies on the mutant VSV ts G16, which has a defective L gene, led to the discovery of a polyadenylation activity for the L protein (Hunt et al., 1984). Furthermore, L is responsible for methylation of the CAP structure (Horikami and Moyer, 1982). Thus L polymerase has a vital role in the processes of transcription and replication during VSV infection.

A/1.2. Subgroups of VSV

Because of these general morphological similarities, members of the VSV group of rhabdoviruses should be compared on a functional scale and in fact have been in several studies. Cartwright and Brown (1972) provided data on the serological relationships between different strains of VSV. By using undissociated virus preparations and sera raised

against infectious virus, cross-neutralization of infectivity was demonstrated for VSV Indiana strains (Indiana, Cocal, and Brazil). Furthermore they observed little cross-neutralization of infectivity between these Indiana subtypes and VSV New Jersey subtypes. In terms of RNA homology, Repik et al (1974) establish another means of contrasting Indiana from New Jersey. These and other studies (Bishop et al., 1974; Repik et al., 1976) indicate that members of the VSV subgroup can be distiguished serologically and biochemically. Protein sequence comparisons show that the G protein of VSV is 50% homologous between Indiana and New Jersey (Banerjee et al., 1984) and NS has as little as 32% homology between subtypes (Gill and Banerjee, 1985). The technique of oligonucleotide fingerprinting of viral RNA was used to further probe the relationships among various VSV subtypes (Clewley et al., 1977). These experiments were extended to analyse not only the difference between Indiana and New Jersey, but the relationship among different isolates of each serotype. Of particular interest is the comparison between the San Juan and Mudd-Summers isolates of VSV Indiana, since these have been used to establish much of the morphological data on VSV. The oligonucleotide fingerprint of San Juan only differs slightly from that of Mudd-Summers (11 oligonucleotides), suggesting that the RNA is highly conserved among these isolates (Clewley et al., 1977). In a study of VSV glycoprotein transport, Gallione and Rose (1985) indicate

some diversity between San Juan and Mudd-Summers. They find that the two isolates differ in approximately 2% of the VSV nucleotide sequence and that about a quarter of these differences affect the amino acids encoded in the VSV genome. With respect to VSV-G, there are nine amino acid sequence differences between Mudd-Summers and San Juan, in the extracellular domain of G. These differences are thought to affect glycosylation and folding of the protein, and ultimately its transport to an infected cell surface (Gallione and Rose, 1985). Such subtle distinctions among these and other VSV subtypes must be taken into account when defining a general mode VSV infection and pathogenesis.

A/2. PATHOGENESIS OF VSV

A/2.1. Cycle of Infection

VSV infection of a particular cell ends with the release of progeny virus and usually death of the host cell. The events leading to this can be summarized as adsorption, uncoating, transcription, translation, replication, assembly and budding (Wagner, 1987). Classically, VSV infection of a monolayer or suspended cell culture begins with a latent period of about two hours, where no viral progeny are detected. Then, after exponential multiplication and release of progeny, the numbers peak at approximately 1000 infectious virions per cell, six to eight hours post-infection (Wagner, 1975).

The first stage of adsorption involves the binding of the VSV envelope to the host cell membrane. The attachment device of VSV is the glycoprotein (G) spike within the viral envelope. If G is removed by proteases, infectivity is reduced more than 10^5-fold (Cartwright et al., 1969; Schloemer and Wagner, 1975). The lipid bilayer of the envelope is also thought to play a role since phospholipase treatment reduces VSV infectivity (Cartwright et al., 1969). Though evidence for a particular host cell receptor is lacking, prolonged treatment of mouse L cells or VERO cells with large amounts of trypsin does not significantly impair VSV attachment, suggesting that readily accessible surface proteins are not receptors for VSV adsorption (Schloemer and Wagner, 1975).

The stages of virus entry (penetration and uncoating) have been reviewed by White et al (1983). Both events occur shortly after adsorption and are energy and temperature dependant. By virtue of quantitative EM studies, VSV particles have been shown to enter cells most often by "viropexis" i.e. intact VS virions enter into phagocytic vesicles, and only rarely by fusion to the plasma membrane (Dahlberg, 1974). Using Semliki Forest Virus, Helenius et al (1980) concluded that enveloped viruses adsorb to the cell surface at the site of histocompatibility antigens and coated pits; injection of the adsorbed virus then appears to occur by endocytosis into coated vesicles. This process also occurs with VSV and at low pH, endocytosis is triggered, leading to

the transfer of nucleocapsid into the cytoplasm (Matlin et al., 1982). Thus intact virus enters the cell via a coated pit, progressing to a coated vesicle, then to large uncoated collecting vacuoles, and finally to secondary lysosomes (Leonard and Miller, 1982).

Once the uncoated nucleocapsid is released into the cytoplasm, it is used as a template for VSV transcription. Transcription occurs without host cell functions and takes place intracellularly in the presence of actinomycin D and cycloheximide (inhibitors of DNA and protein synthesis, respectively) (Huang and Manders, 1972). Parental (input) nucleocapsids are transcribed using a virion-associated RNA-dependent RNA polymerase (Baltimore et al., 1970). This activity involves an N protein encapsidated genomic RNA reconstituted with both the L and the NS proteins (Emerson and Yu, 1975) to transcribe the five monocistronic mRNAs as well as an uncapped, unmethylated 47 nucleotide leader RNA (Colono and Banerjee, 1977). The M protein also regulates transcription when bound to the nucleocapsid, inhibiting the process by about 80% (Wilson and Leonard, 1981).

Each of the mRNAs of VSV excluding the leader RNA sequence is translated immediately after transcription. In fact, the two processes are thought to be coupled. All five VSV proteins are synthesized throughout infection in decreasing amounts relative to the linear genomic sequence N, NS, M, G and L (Hsu et al., 1979). The G protein, as

previously mentioned, is synthesized from mRNA on ER membrane associated polyribosomes leading to transport and insertion in the cell membrane (Knipe et al., 1977; Rothman et al., 1980). The other four VSV proteins are synthesized from monocistronic messengers on cytoplasmic polyribosomes (Morrison and Lodish, 1975). Certain proteins such as NS and to a lesser extent M are phosphorylated (Imblum and Wagner, 1974). The L protein is thought to have kinase activity and possibly regulates the degree of NS phosphorylation (Sanchez et al., 1985). It is the degree of phosphorylation of NS that seems to determine its role in VSV transcriptase activity. Finally, although different mRNAs are equally effective in binding to ribosomes to initiate and elongate polypeptides, N encoding mRNA seems to be more efficiently translated by initiating factors than G message (Lodish and Froshauer, 1977). In general, VSV mRNAs out compete cellular mRNAs for ribosome binding (Lodish and Porter, 1980). This competitive advantage may explain the viral capacity to reduce or inhibit cellular protein synthesis.

The stage of replication is also thought to couple translation and uses the same endogenous polymerase as in transcription (Patton et al., 1983). All full length plus and minus strand RNA molecules (as well as same message and leader RNAs) are encapsidated with N protein (Blumberg et al., 1981). The viral N protein is thought to modulate transcription and replication by its ability to bind to the

5' terminus of leader RNA, thus promoting readthrough of transcription termination signals to allow full length RNA production and assembly into nucleocapsids (Schubert et al., 1982). The critical role of N was further analysed using a monoclonal antibody that reacts with unbound cytoplasmic N, selectively inhibiting genomic replication but not transcription (Arnheiter et al., 1985). A different monoclonal antibody to N inhibits transcription and not replication. Therefore N is thought to exist in two conformational forms that selectively regulate replication and transcription.

Following replication and translation, newly synthesized N, L and NS proteins bind to newly synthesized progeny RNA to form assembled ribonucleoprotein cores of VS virions (Rubio et al., 1980). Independently synthesized M protein then binds to these progeny nucleocapsids resulting in the formation of tightly coiled "skeleton", the final nucleocapsid form in VS virions (Newcomb et al., 1982). At the same time, the VSV G protein is synthesized, glycosylated in ER-Golgi structures, and finally inserted into the plasma membrane (Rothman et al., 1980). The nucleocapsid-M protein complexes appear then to migrate and bind to the cytoplasmic surface of the membrane in a region rich in G protein and amino phospholipids (Zakowski et al., 1981). The tightly coiled nucleocapsid is then enveloped in this G protein converted membrane, leading to budding and release of fully formed and infectious VS

virions (Jacobs and Penhoet, 1982).

A/2.2. Cytopathology

VSV generally causes rapid cytopathic effects in vertebrate and, to some extent, in invertebrate cells. Bablanian (1975) reviewed VSV pathogenesis, which varies among virus strains and serotypes. He suggested that VSV infection resulted in two distinct cytopathic effects. One is a rapid cellular response at high multiplicity of infection, characterized by cells rounding up within an hour and is independent of viral synthetic functions; the other is a slower response thought to require active VSV replication and results in the release of progeny virions from the infected cell (Bablanian, 1975). During the rapid cellular response, VSV evokes an early inhibition of cellular RNA, DNA and protein synthesis (McGowan and Wagner, 1981; Marvaldi et al., 1978). Cells vary in their susceptibility to viral infection, regardless of surface receptors for adsorption (Wagner et al., 1984). Furthermore, inhibition of macromolecular synthesis may differ between cell types even when virus yields are similar. For example, MPC-11 mouse myeloma cells seem more susceptible to VSV shut-off of cellular RNA synthesis than BHK-21 cells or mouse L cells (Weck and Wagner, 1978). VSV induces cytopathic changes in a wide range of mammalian host cells, although some are resistant to killing. The rabbit kidney line DRK-3 appears not to adsorb virus (Chen and

Crouch, 1978) where rabbit corneal RC-60 cells are restricted in VSV growth due only to inhibition of viral RNA replication, since transcription and translation seem to occur (Thacore and Youngner, 1975). Human lymphoblastoid cells of the T cell lineage are permissive for VSV replication, where those of B cell origin are restrictive, possibly due to a failure to inhibit host protein synthesis along with reduced viral transcription (Nowakowski et al., 1973). Rosenthal et al (1986) demonstrated that although cloned murine cytotoxic T lymphocytes were resistant to productive infection by VSV, cloned lines of IL-2 dependent natural killer cells were persistently infected. These and many other studies attempt to decipher the patterns of disease and protection involved in VSV interaction with its host.

A/2.3. Virus-Host Interactions

The economic and veterinary importance of VSV came from outbreaks of a vesicular stomatitis disease in cattle and swine populations that resembled foot-and-mouth disease. Affected animals developed fever, lethargy, decreased appetite and vesicular lesions of the mouth, teats and coronary band of the foot (Hanson, 1970). These vesicles rupture easily, and complete healing takes 7-10 days. Five VSV group viruses (Indiana, New Jersey, Alagoas, Piry, and Chandipura) are known to produce disease in humans (Tesh and Johnson, 1975; Karabatsos, 1985). This disease is characterized by an acute

influenza-like illness, fever, myalgia, headache, and malaise lasting 3-6 days. In general, pathogenesis of VSV Indiana and VSV New Jersey depends on several factors such as virus dose, route of infection, and the age and species of the host (Shope and Tesh, 1987). For example, susceptible cows, swine, guinea pigs, and horses form vesicles if inoculated intralingually with either VSV serotype, but intramuscular inoculation produces inapparent infection and immunity (Proctor and Sherman, 1975). With respect to age, subcutaneous or intramuscular VSV inoculation of newborn mice or hamsters results in a rapid lethal effect on the liver and kidney (Murphy et al., 1975). Older mice and hamsters however generally survive infection with either VSV serotype and develop protective antibodies (Fultz et al., 1982). Injection of VSV Indiana or VSV New Jersey intracerebrally or intranasally into lab rodents is lethal regardless of the animal's age, producing acute necrotizing encephalitis (Murphy et al., 1975). Murine hosts provide a particularly good model to study viral pathogenesis and the mechanisms involved in protection or antiviral immunity. VSV induces both humoral immunity in the form of protective neutralizing antibody (Beatrice and Wagner, 1980) and a cell-mediated immune response via virus specific cytotoxic T lymphocytes (Doherty et al., 1976).

A/3. CELL-MEDIATED IMMUNITY

The vast array of immune responses in mammals can be viewed as the functioning of two complementary processes: humoral immunity, mediated by antibody production and cell-mediated immunity mediated by a number of thymus derived (T) lymphocytes. In humoral immunity, B lymphocytes are the cells responsible for antibody or immunoglobulin production. They bear these immunoglobulin (Ig) molecules on their surface as receptors for antigen. T lymphocytes however express a different antigen recognition structure, the T cell receptor (TcR), on their cell membranes (Pasternack, 1988). Striking differences in antigen reactivity shown by T and B lymphocytes have been discovered in light of their distinct receptors (Germain, 1986). Where B cells are able to bind free antigen such as toxins, bacteria and viruses, T cells recognize antigen on the surface of other cells in conjunction with appropriate surface glycoproteins encoded by the major histocompatibility locus (MHC) (Germain, 1986). This phenomenon known as "MHC restriction" explains, for example, how T cells may react with viral proteins only on the surface of virus infected cells but do not interact with free virions. T cells mediate a number of immune responses such as delayed type hypersensitivity, augmentation of B cell antibody synthesis, cell mediated cytotoxicity and regulation of these processes by T helper (Th) and T suppressor (Ts) cells (Pasternack, 1988). In each case cell-to-cell contact

precedes T cell activation and function. It is the recognition and contact of specific target cells by the effector cytotoxic T lymphocyte (CTL) that initiates T cell mediated cytotoxicity. Studies by Goldstein and Smith (1976) show that this response requires calcium and magnesium ions and takes place in at least three distinguishable phases. The first phase of "conformation" involves cell recognition and the establishment of stable cell contact. This includes recognition of antigen. The second phase is calcium dependent and constitutes the "lethal hit" stage where the target cell is irreversibly programmed for lysis (Goldstein and Smith, 1976). The third phase is that of target cell disintegration where homeostasis breaks down and cytoplasmic macromolecules are released into the incubation medium. This stage no longer requires the continuing contact with effector CTLs (Duke et al., 1983). With respect to time, the first phase occurs rapidly upon CTL-target cell contact, the second phase over the next 15 minutes and the last phase may occur minutes to hours thereafter (Pasternack, 1988).

Cytotoxic T lymphocytes may be defined as thymus derived lymphocytes that specifically recognize and lyse target cells bearing particular foreign antigen and self-MHC proteins or those bearing foreign MHC antigens (Pasternack, 1988). CTLs had been phenotypically defined as CD8 positive human lymphocytes, where CD4 positive cells were Th cells (Reinherz and Schlossman, 1980). Similarly, in the murine system, CTLs

were considered Ly 1^-, Lyt 2^+, and Th cells were Ly 1^+, Lyt 2^- (Cantor and Boyse, 1975). In both systems, CTLs were Class I MHC restricted, and Th cells were Class II MHC restricted. However, more recent studies showing human $T4^+$, $T8^-$ (Meuer et al., 1982b) and murine Lyt 2^-, $L3T4^+$ (Swain et al., 1981) T lymphocytes that are functionally CTLs, prompted a reclassification of these cell types. Consequently, phenotypic markers on lymphocytes now denote not their functional role, but rather which type of MHC molecule is recognized by their T cell receptors. Thus CTL or Th that bind Class I MHC products (membrane proteins expressed on all cells) bear CD8 or Lyt 2 on their surfaces; and T cells that recognize Class II MHC products (found on the surface of a restricted subset of immune response cells such as macrophages, B cells, and some T cells) are CD4 or L3T4 positive (Pasternack, 1988).

The T cell receptor (TcR) was identified using monoclonal antibodies against unique T cell determinants. By immunoprecipitation and gel electrophoresis under reducing and non-reducing conditions, the determinants were shown to be present on an 85 Kd disulphide bonded heterodimer (Allison et al., 1982). Further analysis by two dimensional gels identified a more acidic alpha chain polypeptide and a more basic beta chain polypeptide (Acuto et al., 1983). It is this T cell receptor that gives each CTL its antigenic specificity. As with immunoglobulin molecules, these proteins have variable

and constant domains encoded by specific gene segments joined together by chromosomal rearrangements during T cell ontogeny (Pasternack, 1988). This, along with somatic mutations, accounts for a large repertoire of TcRs able to recognize a large number of antigenic determinants. Additionally, there are other T cell surface molecules involved in cytotoxicity, such as CD3, that are noncovalently associated with the TcR (Meuer et al., 1982a). In murine CTL, there are molecules such as LFA-1 (lymphocyte function-associated antigen 1) (Davignon et al., 1981), LFA-2 (the sheep erythrocyte receptor (Sanchez-Madrid et al., 1982) and a uniquely glycosylated T200 (leukocyte common antigen), that are not bound to TcR, but participate directly in CTL mediated lysis.

A/4. THE CYTOLYTIC PROCESS

A/4.1. Antigen Recognition

With regard to the specificity of T cells for antigens, whether they be naturally occurring proteins or synthetic peptides, the particular form of the antigen recognized has been a controversial matter. In several analyses of T cell function, it was consistently concluded that a denatured form of the antigen served to stimulate a response (Gell and Benacerraf, 1959; Senyk et al., 1971; Thompson et al., 1972; Ishizaka et al., 1975). This antigen recognized by T cells was said to be presented by another cell type functionally known as the antigen presenting cell (APC) (Moller, 1978).

Classically, macrophages, dendritic cells and B cells have been found to play the role of the APC, each cell type expressing Class I and Class II MHC molecules on their surfaces (Hedrick, 1988; Van Voorhis et al., 1983). Inevitably, it was discovered that T cells not only recognize antigenic fragments but also determinants on MHC molecules. Several experiments using Th and CTL populations support this notion. One set of adoptive transfer experiments showed that carrier primed Th cells and hapten primed B cells coming from different mice bearing the same I region gene of MHC (Class II), could cooperate to induce antibody synthesis against the protein complex in a reconstituted mouse (Katz et al., 1973, 1975). If the donor mice were not compatible in their Class II MHC region, no specific antibodies would be produced. Other studies by Zinkernagel and Doherty (1974) demonstrated Class I MHC restriction of CTL populations. These CTLs specifically recognized lymphocytic choriomeningitis virus (LCMV) antigens presented by mouse fibroblasts. Using the four hour ^{51}Chromium release assay of Cerrotini and Brunner (1974), specific CTL activity was found to be significant only when the effector CTLs and the virus infected target cells were of the same H-2 (Class I) haplotype. Therefore, it was clear that the determinant recognized by T lymphocytes depended on the allelic form of Class I or Class II MHC and on the amino acid sequence of an antigen (Hedrick, 1988).

Since T cells were found to recognize denatured or

fragmented antigen, it was thought that the antigen was processed within the APC before presentation to the T cell. In fact, Ziegler and Unanue (1981, 1982) showed that antigen presentation by macrophages required active metabolism and was time dependent. This process occurs within acidified intracellular lysosomes. Substances such as ammonia and chloroquine were used to raise the pH within these lysosomes which caused their dysfunction. This interfered with the ability of macrophages to present antigen only if the substances were added early in the reaction with T cells (Ziegler and Unanue, 1982). Other experiments showed more directly that antigen must be processed. The B cell lymphoma A20-2J serves as an APC for ovalbumin (OVA) specific T cells (Shimonkevitz et al., 1983). If the A20-2J cells first take up OVA and then were fixed with gluteraldehyde, they were able to present antigen despite being metabolically inactive. However, if gluteraldehyde fixation occured before OVA addition, they could not present antigen. More interesting results showed that fixed A20-2J cells can present OVA to some T cell hybridomas if the OVA has been previously fragmented with trypsin, chymotrypsin or cyanogen bromide. Denatured OVA (with SDS) was not recognised. Furthermore, these fixed cells could present synthetic peptides corresponding to specific OVA peptide sequences (Shimonkevitz et al., 1984). Therefore, cleavage of antigen was a necessary requirement for antigen processing and thus for recognition by T lymphocytes. Studies

on the required sequence and conformation of recognized antigen have been reviewed by Hendrick (1988).

Two hypotheses have previously been held regarding the simultaneous recognition of antigen and MHC. The first was that of "altered self" where the TcR recognizes MHC molecules that are modified by bound antigen (Zinkernagal and Doherty, 1977; Matzinger, 1981). The second one, held by Cohn and Epstein (1978) was that of "dual recognition" where the TcR had separate binding sites for each determinant. Later, a model based on interactions between the TcR, MHC and the antigen cytochrome c lended support to the altered self hypothesis. This model proposed that a particular antigen would display different determinants to the TcR depending on the MHC allelic form to which it was bound. This was based on experiments with T cell clones specific for cytochrome c in association with two different Class II MHC alleles (Heber-Katz et al., 1982; Hendrick et al., 1982, Matis et al., 1983). Along with results where the specificity of the antigen recognized by T cell clones was dependent on Class I MHC restriction (Hunig and Bevan, 1982), antigen and MHC molecules were shown to be recognized as one structure by the TcR. More recent evidence from Ashwell and Schwartz (1986) supports this model by testing T cell activity in an antigen dose response curve. Using moth cytochrome c peptide fragment 81-104 bound to MHC Class II, they measured T cell activity as binding of the TcR with the peptide-MHC ligand. More importantly, they

showed that if these cytochrome c peptides were substituted at either position 99 (Lysine to Argenine) or 103 (Lysine to Argenine), two fundamental binding sites were blocked (Ashwell et al., 1986). Position 99 was part of the binding site for the TcR and position 103 was the binding site for the Class II MHC molecules. This data verifies that there is an association of antigen with MHC and a further association of the TcR with this antigen-MHC ligand.

In an attempt to study the conformation of antigenic peptides recognized in association with MHC, Berkower et al. (1986) studied the antigenic properties of sperm whale myoglobin associated with the Class II MHC molecule I-E^d. Briefly, they proposed that the peptide was presented in an amphipathic alpha helical form such that hydrophobic amino acids are lined up on one side to contact the MHC molecule, and the opposite hydrophilic side contacts the TcR. Livingstone and Fathman (1987) used this hypothesis to successfully predict regions within the sperm whale myoglobin protein that could act as antigenic fragments. Furthermore, Rothbard and Taylor (1988) found a pattern, based on the analysis of several antigenic sites, of a charged amino acid or glycine followed by two hydrophibic amino acids. They used this pattern to successfully predict one of the antigenic peptides from the influenza nucleoprotein. In every case, the property of amphipathicity was not essential for TcR binding, but did increase the probability that a peptide interacts with

Class II MHC molecules to stimulate a T cell response.

Other work focussed on the nature of the determinant recognized by CTLs in association with Class I MHC. As mentioned, CTLs are induced to respond to endogenously synthesized foreign antigens, such as with virus infection. Thus CTLs are thought to mostly recognize membrane antigens like viral glycoproteins on a target cell surface in association with MHC. However, CTL specificity studies concerning influenza virus proved otherwise. A majority of the CTL population specific for influenza infected cells were found to respond to the internal nucleoprotein and not to the cell surface hemeagglutinin (Townsend and Skehel, 1984). Nucleoprotein could not be detected on the plasma membrane and since it lacks a signal sequence, it could not have been transported as an integral membrane protein. Therefore, these authors suggested that at least an antigenic portion of the molecules was transported to the cell surface by a different mechanism. One study showed that a population of CTL from H-2^b mice could lyse L cells (H-2^k) cotransfected with D^b and the entire nucleoprotein (498 amino acids long) or with truncated fragments 1-386 or 328-498 (Townsend et al., 1985). This demonstrated that the antigen determinant was located in the overlapping region 328-386 and that transport to the cell surface for recognition by CTL did not seem to require the N and C terminus of the nucleoprotein. Further support involved CTL lysis of target cells incubated with synthetic peptides

derived from certain nucleoprotein sequences, the most efficiently recognized peptide being 366-379 (Townsend et al., 1986b). Therefore it was hypothesized that a degraded form of the endogenously synthesized protein binds to MHC and is transported to the cell surface and this antigenic fragment is recognized specifically by the CTL TcR (Townsend et al., 1986b). X-ray crystallography studies were performed on the Class I MHC molecule (HLA-A2) to examine its structural complexity, how the molecule interacts with foreign antigens and how it is recognized by T cell receptors (Bjorkman et al., 1987). It was discovered that the extracellular portion of HLA-A2 has the following structure: the α_1 and α_2 domains of the MHC molecule form a β-pleated sheet platform topped by two long α-helices. There is a deep groove running between these helices which is thought to be a binding pocket for foreign antigens. Indeed, an unidentified peptide was found to be associated with this pocket. In this manner, the MHC molecule and bound antigen may be recognized by a T cell receptor (Bjorkman et al., 1987).

These studies and evidence for Class II MHC antigen recognition support a model for specific TcR recognition of antigenic peptides bound to surface MHC molecules. Thus, a stable contact may be set up between the effector CTL and its target cell.

A/4.2. Programming for Lysis

The specific interaction between a cytotoxic T lymphocyte

and its target cell results in an irreversible lysis of the latter. A number of studies using cloned CTL and nonspecific natural killer (NK) cell lines have attempted to find the mechanisms behind this lytic process. Zagury (1982) first proposed the idea that the lethal hit of a target cell by its effector was the result of exocytosis of cytolytic molecules from the killer. The origin of these molecules was discovered after a number of CTL and NK cells were examined and found to contain unique organelles referred to as "cytotoxic granules" in their cytoplasm (Podack and Konigsberg, 1984). These electron dense granules seemed to arise from the Golgi apparatus. By EM analysis and other methods, they were not found in non-cytotoxic T lymphocytes. Once a CTL contacts a specific target cell, dynamic alterations are triggered within the cytoplasm of the CTL. Both the Golgi apparatus and the microtubular organizing complex reorient in the direction of contact with the target (Geiger et al., 1982; Kupfer and Dennert, 1984). The functions of these two organelles include protein secretion, suggesting that this reorientation focusses the release of contents from secretory granules onto the target at the point of contact with the killer cell. Yannelli et al. (1986) showed that this action of granule secretion is actual and is accompanied by the appearance of tubular channels on the target cell membrane. If CTL membranes were disrupted by nitrogen cavitation, cytotoxic granules could be recovered by density-gradient centrifugation (Podack and

Konigsberg, 1984; Henkart et al., 1984) and have been shown to independently lyse a number of nucleated target cells at 37°C in the presence of calcium regardless of the antigenic specificity of the CTL from which they were derived. Target cells treated with granules bear transmembrane pores or channels seen just as when they are exposed to intact CTL (Podack et al., 1985). This membrane channel formation then results in the leakage of water, salts, nucleotides and proteins, an irreversible lethal event similar to complement mediated lysis.

Cytotoxic granules have been shown to contain a small number of different proteins that play a role in the lytic process (reviewed by Pasternack, 1988). One of these proteins is Perforin (also known as PFP, cytolysin) which has been isolated from CTL and from cloned lymphocytes that show a high NK-like activity. Perforin has been shown to directly mediate hemolysis and, in the presence of calcium ions, polymerizes to form the tubular pores found on the membranes of target cells (Masson and Tschopp, 1985; Podack et al., 1985). It migrates on SDS-polyacrylamide gels at a molecular weight of 70 to 75 Kd under reducing conditions and 60 to 65 Kd under nonreducing conditions. In a calcium environment, Perforin polymerizes into a ring-like tubular structure with a diameter of 16 nm (Young et al., 1986a). These tubules resist dissociation by SDS and other reducing agents. Calcium is also required for insertion of Perforin into the target

membrane. Young and Cohn (1986) postulate that the calcium binding to Perforin may induce conformational changes such as the unfolding of this protein to expose lipid-binding domains. As mentioned, this type of pore-forming membrane damage is similar to the complement cascade activity of the humoral immune response. In fact, the ninth component of complement (C9) is antigenically related to Perforin (Young et al., 1986b). Furthermore, C9 having a molecular weight of 70-75 Kd (reducing conditions) and 62-66 Kd (nonreducing conditions) is also responsible for the formation of ring-like lesions associated with complement mediated cell lysis (Podack and Tschopp, 1982). Such structural and functional homologies between Perforin and C9 suggest that the two lytic proteins have a common genetic ancestry (Young et al., 1986b). As with antibody induced complement activation, CTL-mediated cytotoxicity is specific for a target cell. Furthermore, there is virtually no nonspecific lysis of bystander cells. Pasternack (1988) suggests that this is partially due to the very rapid polymerization of Perforin upon granule exocytosis. In calcium containing media, Perforin readily aggregates, even with the lack of adjacent membranes. This prepolymerization inhibits its ability to insert into membranes. Therefore, the formation of Perforin channels is limited to within the narrow intercellular space between a target cell and an individual CTL and only this target is lysed.

In addition to Perforin, there are several other lytic

mediators that have a role in cell killing. Several laboratories have isolated a serine esterase from the cytotoxic granules of cloned CTL and NK cell lines (Pasternack and Eisen, 1985; Young et al., 1986a; Tschopp and Masson, 1987). Pasternack and Eisen (1985) have shown that one such 28 Kd serine esterase is expressed at much higher levels in CTL than in helper T cells or resident thymocytes. This enzyme is highly positively charged and is secreted during the cytolytic attack of certain target cells. Its involvement in the cytolytic process has been suggested by the results of inhibition experiments where killing of target cells by cloned CTLs and NK cells was suppressed by protease inhibitors and chymotrypsin or trypsin-ester substrates (Chang and Eisen, 1980; Redelman and Hudig, 1980). When cytolytic granules isolated from cloned CTL lines were preincubated with the irreversible protease inhibitor H-D-Pro-Pre-Arg-chloromethyl ketone, their cytolytic activity was blocked in a dose dependent manner. In fact, if the T lymphocyte serine esterase TSP-1 isolated from these granules was incubated with the same inhibitor, its enzymatic activity was also blocked (Simon et al., 1987). However, since there was no evidence for a direct cytolytic activity of TSP-1 against a panel of normal and tumor target cells, it was thought to play an indirect regulatory role in the cytolytic process (Simon et al., 1986). In terms of the number of such serine esterases present, several studies have been pursued. Masson et al.

(1986) have isolated at least two such enzymes from the granules of cloned CTLs. Using subtractive hybridization protocols to screen cDNA libraries, genes specifically expressed in CTLs or NK cells have been cloned (Gershenfeld and Weissman, 1986). To date, three cDNA clones have been recovered and sequence analysis reveals that these CTL-specific genes encode serine proteases. A number of groups have isolated serine proteases from CTL granules: granzyme A (Masson and Tschopp, 1987); TSP-1 (Simon et al., 1986); SE-1 (Young et al., 1986a); and BLT-serine esterase (Pasternack and Eisen, 1985). Each of these proteases has a molecular weight of 60 Kd, but runs at 30-35 Kd, under reducing conditions, suggesting a dimeric nature (Pasternack et al., 1986). It is not yet known whether individual CTL clones each express only one or a number of serine proteases. Pasternack (1988) postulated that this family of regulatory enzymes may act in a proteolytic cascade to modulate the activity of Perforin in the lytic process, but lack of specific evidence hinders this conclusion. The cytotoxic granules of NK cells have been found to also contain large quantities of negatively charged proteoglycans (MacDermott et al., 1985). These may be involved in modulating the activity of positively charged proteases within the cytolytic granules.

Lymphotoxin (LT) another T cell product has been implicated in target cell destruction (Young and Cohn, 1986). This product has been detected in supernatants of CTL

restricted by Class I or Class II MHC molecules after specific antigen or mitogen stimulation. LT is a glycoprotein, with a molecular weight of 25 Kd, that inhibits the growth of certain LT-sensitive cells in a standard three day assay (Schmid and Ruddle, 1988). However, it is difficult to find a role for LT in the lytic process for several reasons. Firstly LT is released by CTL with very slow kinetics. Also, unlike CTL-mediated killing, lysis of cells by LT is at a slow rate and does not involve specificity for antigen. However, if soluble LT in supernatant is taken up by cells and is detectable in their cytoplasm, rapid target cell destruction is triggered (Schmid et al., 1986). It is presumed that LT is transferred from CTL into the cytoplasm of target cells via the transmembrane Perforin pores, and thus represents another means of target cell destruction (Schmid et al., 1986). The implications for this LT-mediated lysis are discussed below.

A/4.3. Target Cell Lysis

The third stage of cell mediated cytotoxicity after the "lethal hit" stage involves "effector cell-independent" target cell disintegration. Here, as mentioned, homeostasis breaks down and cytoplasmic macromolecules are released into the incubation medium (Young and Cohn, 1986). This aspect of CTL-mediated lysis closely resembles lysis by complement cascade in that both appear to involve transmembrane pore formation (Pasternack, 1988). However, when murine target cells are lysed by antibody and complement (or by hypotonic swelling)

the nuclear contents remain intact (Russell et al., 1982). Immediately after a murine CTL conjugates with a murine target cell, the nuclear chromatin of the target undergoes gross morphological changes (Russell et al., 1982). This involves the initiation of a rapid DNA fragmentation process in the target cell nucleus. Duke et al. (1983) described this process as "apoptosis" or "programmed cell death" which ultimately results in the complete breakdown of DNA into small fragments of approximately 180 base pairs. They found that this fragmentation within target cell nuclei precedes ^{51}Chromium release in a standard assay, appears to be mediated by a specific endonuclease, and is inhibited by zinc ions and not by protein synthesis inhibitors (Duke et al., 1983). Furthermore, this process is thought to be related to cell mediated cytolysis since freeze-thawing, heating, or antibody-complement mediated lysis do not yield DNA fragments. Interestingly, LT treated cells do undergo chemical fragmentation of this sort (Schmid et al., 1986). Also murine natural killer cells were shown to cause DNA fragmentation in murine YAC-1 target cells (Christiaansen and Sears, 1985). However, this process has been shown not to occur significantly in human tumor target cells lysed by human CTL and NK lymphocytes. It has been suggested that fragmentation is not a necessary aspect of lymphocyte induced lytic processes (Christiaansen and Sears, 1985). Therefore, DNA fragmentation represents a species specific process occurring

in murine cells upon lysis by CTLs or NK cells. Christiaansen and Sears (1985) propose that the DNAse activity is probably internally activated in the murine target rather than delivered by the effector cell since, human killer lymphocytes selectively cause DNA fragmentation in murine but not human target cells. This activation may then be mediated by some element (such as LT) that is specifically found in CTLs and NK cells. These events following specific antigen recognition (in the case of CTLs) and programmed lethal hit, contribute to the overall lytic processes of the effector cell.

A/5. THE IMMUNE RESPONSE TO VSV

VSV induces both a humoral and a cell-mediated immune response in infected animals. Exhaustive studies have measured and characterized these responses, using the mouse as a model system.

The major substance that protects animals against VSV infection is neutralizing antibody. This humoral response appears within ten to twelve days after VSV infection (Wagner, 1974). The antiviral antibodies generated during the humoral immune response are directed to two major antigens known as "group specific" and "type specific" (Brown and Cartwright, 1966). Group specific antibodies were later found to be directed to the N protein and type specific to the G protein (Kang and Prevec, 1970; Cartwright and Brown, 1972). As such, VSV was divided into its two major serotypes, Indiana and New

Jersey. Antibodies against the G protein of either serotype were found to be non-crossreactive (Cartwright and Brown, 1972). Kelley et al (1972) identified the type specific antigen (VSV-G) as the protein giving rise to and reacting with neutralizing antibody. Lefrancois and Lyles (1983a) examined the glycoproteins of VSV Indiana and VSV New Jersey to further define this antibody-mediated neutralization. Using a panel of monoclonal antibodies against VSV-G in competitive binding assays, they found that the glycoproteins of either serotype possess at least four distinct antigenic determinants involved in virus neutralization. The capacity of monoclonals against G of either serotype to protect mice against the neuropathic and lethal effects of VSV was also studied (Lefrancois, 1984). Mice were protected only if neutralizing antibodies were administered 24 hours prior to or simultaneous with the virus (of the same serotype) and not after virus challenge. Along with these neutralizing antibodies, infected mice produce non-neutralizing antibodies that appear to be cross-reactive between the two VSV serotypes (Lefrancois and Lyles, 1982).

The aspects of cell mediated immunity to VSV have previously been reviewed (Zinkernagel et al., 1978a). It was discovered that VSV infection of mice generated CTLs specific for both a viral antigen and either the K or D locus or both of the H-2 histocompatibility complex (Doherty et al., 1976). Rosenthal and Zinkernagel (1981) examined the ability of

various mouse strains to generate CTL against VSV. Their results showed that H-2^k mice were unable to generate VSV specific CTL (to either serotype) despite the ability to generate a humoral response. Both H-2^b and H-2^d were capable of generating a complete (humoral and cell-mediated) immune response to VSV. Based on studies using ts mutants of VSV, the VSV glycoprotein was previously thought to be the major target of CTLs (Hale et al., 1978; Zinkernagel et al., 1978b). In contrast to the non-crossreactivity of antibodies specific for this viral antigen, Rosenthal and Zinkernagel (1980) found that cells infected with either VSV Indiana or VSV New Jersey were specifically lysed by effector T cells generated by either serotype. To analyse the basis of this T cell crossreactivity, CTL clones were generated in vitro against one serotype (Indiana) and examined for ability to lyse syngeneic cells infected with VSV New Jersey. Crossreactivity was established along with results where non-crossreactive VSV-G specific antibodies could not block anti-VSV CTL (Rosenthal et al., 1983). Therefore, it was suggested that serotype specific neutralizing antibodies and specific CTL recognize distinct epitopes of the VSV glycoprotein. Recent results using cell lines expressing VSV genes have concluded that the specificity of anti-VSV CTL is predominantly toward the nucleocapsid (N) protein (Puddington et al., 1986). Yewdell et al. (1986) examined CTL specificity by using recombinant vaccinia virus containing copies of either VSV-G

or VSV-N genes. They compared the recognition of VSV-G and VSV-N and found that N serves as the major target antigen for anti-VSV CTL. These and other studies may thus lead to a clearer understanding of the cell-mediated immune response to VSV.

A/6. RECOMBINANT VIRUS VECTORS

With the occurrence of several VSV induced disease outbreaks in livestock, it became necessary to develop some type of effective vaccine against VSV. Olitsky et al. (1928) first reported that intramuscular inoculation of cattle with live VSV resulted in a lack of visible lesions and thus resulted in immunity to local challenge. Later developments included the use of live unmodified VSV, inactivated virus and vaccines composed of VSV subunits (such as the glycoprotein G) (Lauerman, 1967; Holbrook and Geleta, 1957).

The most recent vaccine involves the incorporation of the gene encoding VSV-G into vaccinia virus (Mackett et al., 1985). The gene is taken up by homologous recombination into a vaccinia virus vector, within its thymidine kinase (TK) gene, and as such is under TK transcriptional and translational control. This recombinant virus vaccine was used to successfully immunize mice and cattle against VSV (Mackett et al.,1985). Vaccinia virus vectors have been used as expression vectors to analyse and detect immune responses toward particular antigens. Mackett et al. (1985) measured

the humoral response of neutralizing antibody production. These expression vectors have also been used to study VSV specific cell-mediated immunity. In this regard, Yewdell et al. (1986) used recombinant vaccinia viruses to measure and compare the CTL response against VSV-G and VSV-N.

Other virus vectors have been used to study immunity and protection against a number of antigens. As outlined by Schneider et al. (1989), the usefulness of a recombinant virus vector depends on such factors as host range, stability of the vector, ease of administration, site of replication and the replicative biology of the vector. The human adenovirus type 5 (Ad5) has been often used to achieve high level expression of a number of foreign inserted genes in vivo. Because it is well characterized in terms of its molecular biology, Ad5 has proven suitable as a recombinant virus vector. Johnson et al. (1988) constructed Ad5 based vectors containing and expressing coding sequences for the herpesvirus type 1 (HSV-1) glycoprotein gB, to study the immunological properties of adenovirus vectors. Also, Ad5 vectors containing human immunodeficiency virus type 1 (HIV-1) genes (coding for envelope proteins) have been used to characterize these proteins and analyse the potential of such vectors as live vaccines against HIV-1 infection (Dewer et al., 1989). Similar studies of immune response involves recombinant adenovirus vectors carrying the hepatitis B virus surface antigen coding sequence (Morin et al., 1987). Finally,

adenovirus vectors have been developed that carry the gene encoding VSV glycoprotein of the Indiana (San Juan) serotype (Schneider et al., 1989). VSV-G serves as a model for evaluating adenovirus-mediated expression and the induction of humoral immune responses in a variety of hosts. It therefore was possible to use this recombinant adenovirus vector to study the cell mediated immune response against VSV.

A/7. THE PURPOSE OF THIS STUDY

The recombinant human adenovirus AdG12 has been previously used as a model to demonstrate the host range of infection and protein expression by Ad vectors. Along with human HeLa cells, AdG12 infected bovine, canine and murine cells were all shown to express VSV glycoprotein (Schneider et al., 1989). Furthermore, AdG12 has been examined for its effectiveness in inducing immune responses in several species. Studies by Prevec et al. (1989) showed that calves, pigs, dogs and mice infected with AdG12 all produced high titres of VSV-neutralizing antibodies in their sera. The potential use of recombinant adenoviral vaccines for the study of antiviral immune responses has thus been considered.

Along with induction of humoral anti-VSV response, it was necessary to examine the use of AdG12 in the induction of cell-mediated responses against VSV. As reviewed by Zinkernagel and Rosenthal (1981), cell-mediated responses such as those of CTL's may differ from humoral responses with

respect to antigenic determinant specificity. Recent studies have focussed on the antigenic specificity of anti-VSV CTL in mice using vaccinia recombinant viruses and cells transformed to express VSV proteins (Puddington et al.,1986; Yewdell et al.,1986). The following studies attempted to confirm these investigations using the recombinant adenovirus vector AdG12.

AdG12 also provided a model to demonstrate adenovirus infection and expression of inserted foreign genes in a number of murine cell lines. Cell lines that could be productively infected with AdG12 and expressed VSV-G were used as targets to measure anti-VSV cell-mediated immune responses in Balb/c and C57Bl/6 mice. Furthermore, the kinetics of anti-VSV CTL production were investigated in mice primed with AdG12.

In this manner, these studies intended to provide a murine model for the use of Ad recombinant vectors to induce cell-mediated immunity and serve as potential vaccines.

B/. MATERIALS AND METHODS

B/1. CELL LINES

B/1.1. Suspended Cell Lines

P815 is an H-2^d mastocytoma cell line derived from DBA/2 mice. EL-4 is an H-2^b thymoma cell line derived from C57Bl/6 mice. Both these cell lines were tested for their ability to serve as targets in ^{51}Cr release assays. YAC-1 is a subline of the YAC lymphoma induced in A/Sn strain mice by Maloney leukemia virus (Keissling et al, 1975). It was used as a ^{51}Cr labelled target cell to indicate murine natural killer (NK) activity.

These suspension cell lines were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal calf serum (FCS; Gibco), 10 mM L-glutamine, 0.075% (w/v) sodium bicarbonate, 10 mM N-2-hydroxyethyl piperazine-N'-2-ethanosulfonic acid (HEPES), 100 ug/ml streptomycin and 100 U/ml penecillin.

B/1.2. Attached Cell Lines

LTA (H-2^k), a subline of parental L cells, is a continuous line originally derived from connective tissue of a C3H/An mouse (Earle, 1943). Z4 (H-2^k) cells are a subline of L cells that constituitively express the herpes simplex virus immediate-early protein ICP4 (Persson et al, 1985). The PAK (H-2^b) and B10.D2 (H-2^d) lines were derived from 20-methyl-

cholanthrine induced tumours (Simrell and Klein, 1979). These murine fiboblast cell lines were used as targets in ^{51}Cr release assays. CL-7 ($H\text{-}2^b$), a similarly derived line, was also tested as a target. Baby hamster kidney cells (BHK-21) and African green monkey cells (Vero) were obtained from the American Type Culture Collection (ATCC, Rockville, MD.).

These attached cell lines were maintained in alpha-minimal essential medium (MEM; Gibco) supplemented with 10% FCS, 10 mM L-glutamine, 0.075% (w/v) sodium bicarbonate, 10 mM HEPES, 100 ug/ml streptomycin and 100 U/ml penecillin.

B/2. VIRUSES

B/2.1. Vesicular Stomatitis Virus

Two variants of vesicular stomatitis virus (VSV) serotype Indiana were used in these studies. The first, Mudd-Summers, was originally obtained from Dr. D. Summers and used as multiply cloned stocks (Rosenthal and Zinkernagal, 1980). The second variant, San Juan, was originally obtained from vesicular tongue epithelium of an infected steer on the San Juan Indian reservation near Espanola, New Mexico, in 1956 (Clewly et al, 1977).

B/2.1.1. Propagation and Titration of VSV

VSV was grown in BHK_{21} cells according to Rosenthal and Zinkernagal (1980). Briefly, confluent monolayers of BHK_{21} cells in 150 cm^2 polysterine tissue culture flasks (Corning, Corning, NY) were infected with VSV at a multiplicity of infection (MOI) of 0.1 plaque forming units per cell. The flasks were incubated at 37°C for 45-60 minutes to allow virus absorption, refed with fresh medium containing a reduced amount of FCS (5%), and then placed in a humidified incubator at 37°C for 24 hours. Virus was harvested by freeze-thawing the flasks once, collecting virus-containing supernatants, and centrifuging at 1500 rpm for ten minutes to clear cellular debris. These virus stocks were aliquoted and frozen at -70°C until needed.

VSV stocks were titred by plaque assay on Vero cells as previously described (Holland and McLaren, 1959). Polysterine 6-welled culture dishes (35mm diameter wells) (Corning) were seeded with $5x10^5$ Vero cells per well. When the cells reached confluency, the medium was aspirated off and they were inoculated with 0.2 ml of serial 10-fold dilutions of virus-containing supernatant per well. The dishes were incubated for 45-60 minutes at 37°C and rocked periodically. Concurrently, a solution of 2% agar (Bethseda Research Laboratories, Bethseda, MD) was melted in boiling water, diluted 1:5 with medium, and kept at 43°C. After virus absorption, the dilutions were aspirated off and 4 mls of the

agar-medium solution was added to each well. The agar solidified at room temperature and the plates were incubated for 24 hours at 37°C. The cells were then fixed and stained with crystal-violet stain to count plaques, and these were calculated as PFU of virus per ml of stock supernatant.

B/2.2. Recombinant Adenovirus Vector AdG12

The recombinant human adenovirus (Ad) vector containing the structural gene of VSV (G) glycoprotein (Indiana-San Juan) was generously provided by Dr. L. Prevec. The promoter and poly(A) addition sequences from herpes simplex virus type 1 thymidine kinase (TK) were added to the glycoprotein gene and this construct was inserted into the early region 3 (E3) of human Ad type 5, in the same orientation as E3, generated according to Schneider et al. (1989).

B/2.2.1. Propagation and Titration of AdG12

AdG12 was grown in HeLa cells as described (Schneider et al, 1989). HeLa is an epithelial-like cell line derived from a human cervical carcinoma (Gey et al,1952). Subconfluent monolayers of HeLa cells in 175 cm^2 flasks (Corning) were infected with AdG12 at an MOI of 1.0 PFU per cell. After 45-60 minutes of virus absorption at 37°C, the virus was aspirated off and MEM medium (Gibco) with 5% FCS (Gibco) was added to the cells. These cells were then placed at 37°C for

4-5 days. By this time, virus induced cytopathic effects (CPE) had taken place and the flasks were freeze-thawed once. The virus containing supernatant was collected and 10% (v/v) glycerol (BDH Chemicals, Toronto, Ontario) was added to keep the virus particles intact during freezer storage. This stock solution was aliquoted and stored at -70°C for later use.

Titration of AdG12 was carried out on KB cells, a human cell line derived from an epidermoid carcinoma (Eagle, 1955). Briefly, 6-well polysterine plates (Corning) containing subconfluent layers of KB cells were inoculated with serially diluted virus stock as described earlier. After virus absorption, the cells were overlayed with 4ml of an agar solution per well, prepared as follows. A 100 ml solution of MEM containing 5% FCS and 1% agar (BRL) was supplemented with 1.6 ml of 2.5 mM $MgCl_2$ (BDH). The plates were incubated at 37°C in a humidified incubator for 5-6 days, at which time, 1 ml of a second overlay was added to each well. This overlay was prepared by adding 5 ml of a 2% agar solution and 1% (v/v) neutral red dye (Gibco) to 20 ml of MEM medium. Plaques were counted 24 hours after incubation at 37°C, to determine the titre of the AdG12 stock.

B/2.3. Adenovirus type 5

Wildtype adenovirus (Ad5), generously provided by Dr. P.E. Branton, was used as a control in expression and ^{51}Cr release assays. A mutant form Add1E3, was created by removing

the Xba1 D fragment of Ad5, which contains part of the non-essential E3 region (Berkner and Sharp, 1983). This construct was also provided by Dr. L. Prevec and was used as a control in these experiments.

B/3. VIABILITY OF CELLS UPON VIRUS INFECTION

The growth pattern of various suspended and attached cell lines was analyzed, comparing uninfected cell populations to those infected with wildtype VSV and AdG12. Approximately 5 x 10^6 cells were infected with VSV at an MOI of 5.0, AdG12 at an MOI of 15.0-20.0, or wildtype Ad5 at an MOI of 15.0-20.0. The infected cell populations were then incubated at 37°C for 45-60 minutes to allow virus absorption. An uninfected control population for each cell type studied was also incubated. The tubes were then centrifuged at 1500 rpm for five minutes to repellet the cells. Adherent cells were plated on 100 mm diameter polysterine tissue culture dishes (Corning) in MEM growth medium and incubated at 37°C for various times up to 36 hours. Likewise, suspension cells were resuspended in RPMI growth medium and incubated. Cell populations were analyzed at specific time intervals every six hours and the number of living cells was determined by trypan blue dye exclusion. Adherent cells were resuspended by washing off growth medium and then incubating the cells in 1.0 ml of a 1X solution of trypsin-Disodium Ethylenediaminetetraacetate (EDTA) (Gibco). The trypsin is

then neutralized with 4.0 ml of medium. An aliquot of 50 μl was taken from each 5.0 ml solution of adherent or suspension cell population and mixed with 50 μl of trypan blue dye (Gibco). The cell population was then counted under 10X magnification using a hemocytometer (Reichert, Buffalo, NY), such that dead cells were stained with blue dye and living cells remained unaffected.

B/4. EXPRESSION OF VIRAL PROTEINS FOLLOWING INFECTION

B/4.1. Infection and Labelling of Various Cell Lines

Adherent and suspended cell lines were infected with wildtype VSV, AdG12, or wildtype Ad5 as described above. The expression of viral specific proteins was determined in the following manner, as described by Bell et al. (1984). At various time points post-infection, 5 x 10^6 cells (of each cell type) were pelleted in 15 ml centrifuge tubes (Corning). Cells were washed once with warm phosphate-buffered saline (PBS) and then incubated for 60 minutes in JOKLIK modified minimal essential medium minus methionine plus [^{35}S] methionine (ICN Biochemicals, St. Laurent, Quebec) at approximately 50 μCi/ml. The labelled cells were then pelleted, washed three times with PBS, and freeze-thawed once. Cell extracts were prepared in RIPA buffer which contains 50 mM TRIS (Sigma, St. Louis, MO) at pH 7.2, 0.15 M NaCl(BDH), 1% (v/v) Triton X-100 (Sigma), 1% (w/v) deoxycholic acid sodium salt (NaDOC; Sigma),

0.1% (w/v) sodium dodecylsulfate (SDS; BIORAD, Richmond, Calif.) and 100 KIU/ml Aprotinin (Sigma).

B/4.2. Immunopreciptation of Viral Proteins

The 1.0 ml [^{35}S]-labelled extract was then prepared for immunoprecipitation of viral proteins as described (Bell et al, 1984). The extract was mixed with 20 µl of antiserum and 250 µl of protein A-Sepharose CL 4B beads (Pharmacia, Uppsala, Sweden) and then incubated with rotation at least four hours at 4°C. After absorption, the beads were centrifuged at 1000 rpm for one minute and the supernatant was removed. Cold RIPA buffer was used to wash the beads four times, prior to analysis on sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

A monoclonal antibody 60-4 of the immunoglobulin class IgG1,isolated from hybridoma clone 60-4 according to Rosenthal et al,(1983), was used to specifically immunoprecipitate surface G protein of VSV. A second polyspecific anti-VSV antibody was needed to enhance this precipitation. The antiserum, H2-19 is a mouse monoclonal antibody, isolated according to Rowe et al, (1984) and used to specifically immunoprecipitate the Ad5 DNA binding 72K early region 2 protein, thus confirming adenovirus infection.

B/4.3. Discontinuous SDS-Polyacrylamide Gel Electrophoresis

Sepharose beads with attached immunoprecipitated viral

proteins were resuspended in buffer which contained 0.5 M TRIS (Boehringer-Manheim Biochemicals, Indianapolis, IN) at pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol (BDH), 1% ß-2-mercaptoethanol (Sigma), and Bromophenol blue dye (BIORAD). Approximately 50 µl of this buffer was added to each sample and the samples were placed in boiling water for 3-5 minutes, to dissociate protein from the beads.

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970). A stock acrylamide solution was prepared consisting of 30% (w/v) acrylamide (BIORAD). A Running gel buffer of 3M TRIS at pH 8.9 and a Stacking gel buffer of 0.5M TRIS at pH 6.7 were also prepared. These solutions were used to produce a 10% polyacrylamide gel in the following manner. A Running gel was prepared by mixing 33.3 ml of the acrylamide solution, 12.5 ml of the Running gel buffer, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulfate (BDH), 0.05% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED; BIORAD) and double distilled water to a total volume of 100 ml. This mixture was poured between two glass plates and allowed to polymerize for 1-2 hours. The Stacking gel was prepared by adding 1.25 ml acrylamide solution, 1.25 ml Stacking gel buffer, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate, 0.2% (v/v) TEMED (BIORAD) and double distilled water to a total volume of 10 ml. The Stacking gel was poured on top of the Running gel, and ploymerized around a plastic comb such that wells were created within the gel. Once the

gel was fully polymerized, 40 µl of each sample (sample buffer with proteins) was added to the individual wells, and electrophoresis was carried out at 50-70 V overnight. A tank buffer with 0.632% (w/v) TRIS, 0.399% (w/v) glycine (BIORAD) and 0.1% (w/v) SDS at pH 8.9 was used for this process. Once the Bromophenol blue dye front (withing the sample buffer) reached the bottom of the gel, the gel was removed, fixed in methanol:water:acetic acid (50:50:7) and dried onto Whatman filter paper (Whatman Inc., Clifton ,NJ) by low-pressure dessication at 100°C. Frequently, the gel was soaked in Enlightning (NEN) (200 ml for 30 minutes) prior to drying, to enhance the intensity of protein banding. The dried gel was exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) for the appropriate length of time.

B/5. GENERATION OF CELL-MEDIATED IMMUNE RESPONSES

B/5.1. Primary Cell-Mediated Immune Responses

Mouse strains used in these experiments were Balb/c (H-2^d), C57Bl/6 (H-2^b) and CBA/J (H-2^k), all purchased from Jackson Laboratories (Bar Harbour, MI) and used between 6 and 12 weeks of age. They were housed in polycarbonate wire top cages and supplied with Purina mouse chow and water ad libitum.

Mice were primed by intravenous (i.v.)injection of approximately 2.0 x 10^7 PFU of wildtype VSV, AdG12, wildtype Ad5, or AddlE3. The mice were sacrificed six to nine days

post-infection by cervical dislocation, and their spleens were excised. A single cell suspension was prepared by passing the spleens through a wire mesh grid. These splenocyte populations were then assayed for cytotoxic activity.

B/6. ASSAY FOR CELL-MEDIATED CYTOTOXICITY

Cytotxicity was measured against ^{51}Cr labelled target cells. To measure virus specific lysis, adherent target cells were infected with wildtype VSV at an MOI of 5.0 three hours before the assay. The cells remained adherent during infection. Other adherent targets were infected with AdG12, wildtype Ad5, or Add1E3 at an MOI of 15.0, thirty-six hours before the assay. Target cells (usually $1x10^7$) were then labelled with 250 μl of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) in a volume of 1.5 ml for the last 90 minutes before the assay, while still attached to 100mm tissue culture dishes (Corning). After labelling, the various target cells were washed four times in MEM with 10% FCS and incubated with effector spleen cells in 96 well flat-bottomed microtiter plates (Nunclon, Denmark). Typical effector:target cell ratios were 40:1, 12:1 and 4:1. Natural killer (NK) activity was measured against ^{51}Cr labelled YAC-1 targets at effector:target ratios of 100:1 and 25:1. The assay proceeded for six hours. At this time, 100 μl of supernatant from each well was removed, pipetted into a 6x50 mm borosilcate glass

tube (Kimble, Owens, IL), and counted in a gamma counter to determine release of ^{51}Cr in counts per minute (cpm). The minimum (background) release of ^{51}Cr from target cells was determined by incubating them in medium alone. The maximum ^{51}Cr release was determined by incubating target cells in 1N hydrochloric acid (HCl). All test wells (minimum, maximum and effector/target samples) were assayed in duplicate. The percent spontaneous release of ^{51}Cr was calculated as:

$$\frac{\text{Average minimum cpm (medium alone)}}{\text{Average maximum cpm (HCl alone)}} \times 100$$

Acceptable values were generally less than 30%.

The percent specific ^{51}Cr release was defined as:

$$\frac{\text{Average test sample cpm - Average minimum cpm}}{\text{Average maximum cpm - Average minimum cpm}} \times 100$$

B/7. DEPLETION OF SPECIFIC CELLS WITHIN EFFECTOR CELL POPULATION

B/7.1. Depletion of Adherent Cell Population

A single cell suspension of spleen cells from either uninfected or infected mice was depleted of adherent cells in the following manner. Each population of splenocytes was plated in 5-10 ml of MEM medium on 100 mm tissue culture dishes (Corning) for two hours at 37°C in a humidified

incubator. During this period, adherent cells such as macrophages settled and attached to the plastic surface. Subsequently, the medium, with non-adherent cells was collected, the cells were counted, and were used in cell mediated cytoxicity assays described above.

B/7.2. Assay for Depletion of T Cells

Single cell suspensions of splenocytes from Balb/c ($H\text{-}2^d$) mice were depleted of T cells by the following method. Spleen cells were first treated with anti-Thy 1.2 antibody. Two types of antibodies were used in separate experiments. The first was HO-13-4 supernatant kindly provided by Dr. D. Sneider of the National Institute of Health (NIH). HO-13-4 is a hybridoma that secretes monoclonal alloantibodies against determinants encoded by the Thy.1.2 locus (Marshak-Rothstein et al, 1979). For every 4×10^7 spleen cells, 2 ml of HO-13-4 and 8 ml of RPMI medium were incubated on ice for 30-45 minutes. The other antibody used was an anti-Thy 1.2 monoclonal antibody from Cedarlane Laboratories Ltd. (Ontario, Canada). The lyophylized antibody was reconstituted in 1 ml sterile distilled water, and mixed at a 1:20 ratio with spleen cells in RPMI medium (1×10^7 cells/ml). This solution was also incubated for 30-45 minutes on ice. These anti-Thy 1.2 treated cells were then centrifuged at 1500 rpm for 5 minutes, and the supernatant was discarded. The cells were resuspended

in diluted Cedarlane LOW-TOX-M Rabbit Complement (Ontario, Canada). The lyophylized complement was first reconstituted in 1 ml distilled water, and then mixed with 9 ml of warmed (37°C) RPMI medium just prior to use. The spleen cells were treated at a concentration of 5×10^7 cells/ml of complement, for 45 minutes at 37°C in the incubator. The cells were then centrifuged, washed with warm PBS, counted and used as effectors in cell mediated cytotoxicity assays.

C/. RESULTS

C/1. Infection of Murine Cell Lines with an Adenovirus Recombinant Vector

The murine cell mediated immune response against a particular virus has been generally measured using the standard ^{51}chromium release assay of Cerottini and Brunner (1974). In this method, cytotoxic T lymphocyte (CTL) responses are measured against infected and uninfected target cells. Established murine suspended cell lines such as P815 and EL-4 have been previously used to measure specific responses against vesicular stomatitis virus (VSV) in an H-2^{d} and an H-2^{b} system respectively (Rosenthal and Zinkernagel, 1981). Typically, cells were infected at an MOI of 5 for four to six hours in order to establish infection and expression of VSV proteins. Adherent cell lines such as B10.D2 (H-2^{d}), PAK (H-2^{k}) and LTA (H-2^{k}) have also been used as targets to measure CTL responses against VSV and HSV.

The following studies have attempted to measure the primary cell-mediated immune response against the adenovirus type 5 recombinant vector, AdG12, which contains the structural gene of the VSV glycoprotein (G). In order to do so, it was first necessary to determine which murine cell lines could be infected with AdG12 and could express VSV-G. In this respect, these lines could be used as target cells in ^{51}Cr release assays.

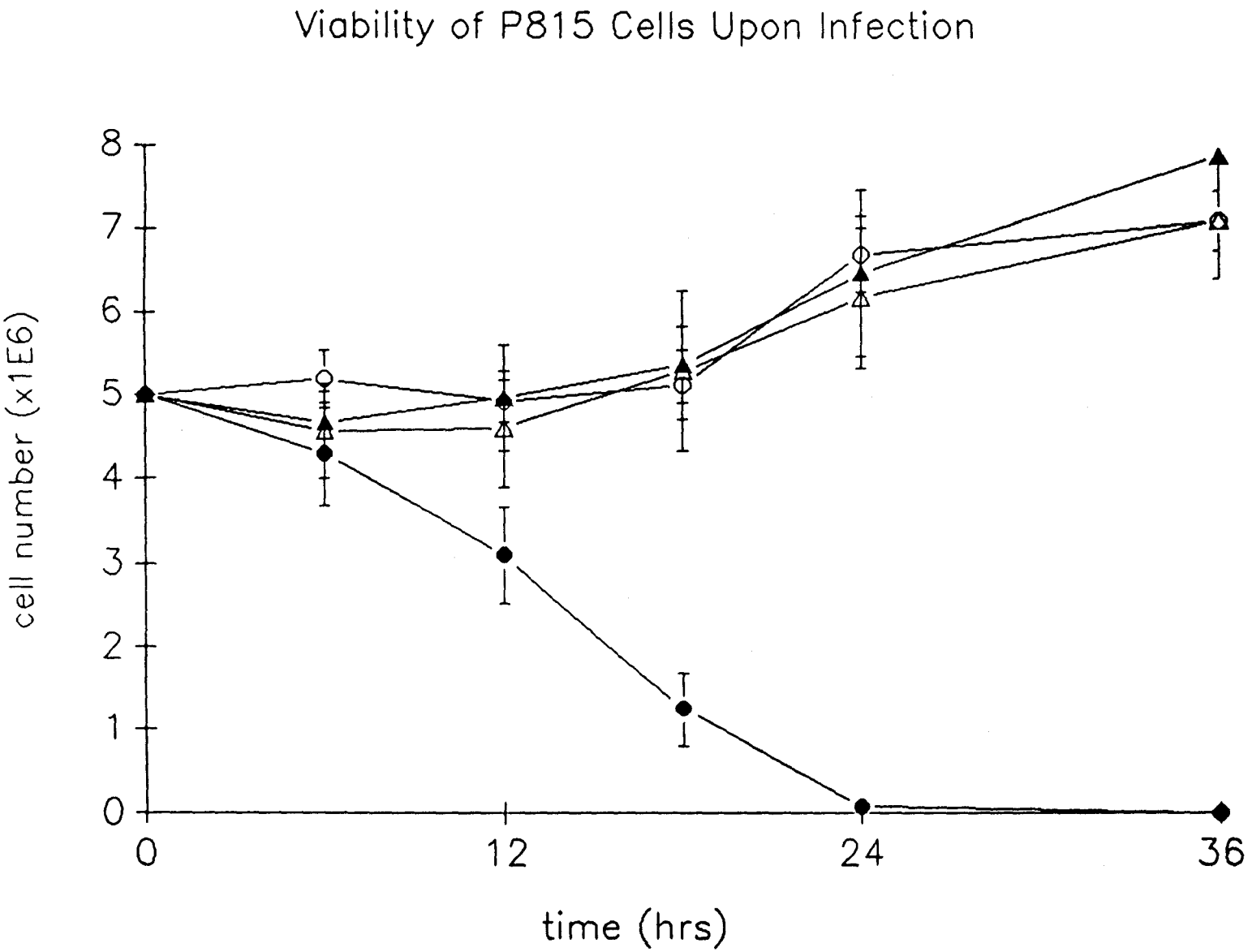
C/1.1 Viability of Murine Cell Lines Upon Virus Infection

Since P815 cells have been commonly used as syngeneic targets for Balb/c (H-2^d) effectors in ^{51}Cr release assays, these cells were tested for infectibility with adenovirus. The P815 cells were first aliquoted into groups of 5 x 10^6 cells and then incubated at 37°C either uninfected, infected with VSV Indiana - Mudd Summers (MS) at an MOI of 5, with adenovirus type 5 (Ad5) or with the AdG12 recombinant. Adenovirus were used at an MOI of 15 to 20. Viability of these cells was then measured from 0 to 36 hours post-incubation using the method of Trypan blue exclusion. This data is illustrated in figure 1.

After 36 hours, the uninfected population of 5 x 10^6 P815 cells increased slightly in number. In contrast, P815 cells infected with VSV_{MS} demonstrated decreased viability and, following 24 hours of infection, there were no living cells present. P815 cells inclubated with either wildtype Ad5 or AdG12 showed the same pattern of increased viability as uninfected cells over the 36 hour period. Thus, up to 36 hours, P815 cells incubated with either Ad5 wt or AdG12 did

Figure 1: Viability of P815 ($H\text{-}2^d$) Cells Upon Virus Infection

The $H\text{-}2^d$ suspended cell line P815 was tested for infectibility with various virus isolates in the following manner. Groups of approximately 5×10^6 P815 cells were incubated alone (○) or with either VSV_{ms} at an MOI of 5 (●), wildtype Ad5 at an MOI of 15 (△) or with the adenovirus recombinant AdG12 at an MOI of 15 (▲). These populations were each incubated at 37°C for 36 hours. During this incubation, cells were counted approximately every 6 hours to determine the number that survived in the presence or absence of virus. Using trypan blue dye, it was possible to determine if cells were subjected to CPE due to virus infection. Results shown on this graph are an average of 3 experiments ± standard deviation (illustrated by error bars).


Viability of P815 Cells Upon Infection
cell number (x1E6)
8
7
6
5
4
3
2
1
0
0
12
24
36
time (hrs)

not appear to undergo the cytopathic effects (CPE) that occurred with VSV_{MS} infection.

Figure 2 shows similar results using the $H\text{-}2^b$ murine suspended cell line EL-4. These cells also showed increased viability of uninfected cells and those incubated with Ad5 or AdG12 for up to 36 hours. In contrast, EL-4 cells infected with VSV_{MS} displayed CPE resulting in decreased viability.

Viability assays were also conducted using murine adherent cell lines. Results using the $H\text{-}2^d$ cell line B10.D2 are illustrated in figure 3. Once again, uninfected B10.D2 cells increased in cell number more than 100% over a 36 hour period, whereas viability of B10.D2 cells infected with VSV_{MS} decreased due to CPE. Interestingly, if B10.D2 cells were incubated with 15 pfu per cell of AdG12 or Ad5 wt, viability increased from 0 to 18 hours, but then declined from 18 to 36 hours. These results strongly suggest that B10.D2 cells were infectible with either Ad5 or AdG12 and, after 18 hours of infection, were lysed due to CPE.

The results in figure 4 indicate this same viability pattern using the $H\text{-}2^k$ cell line LTA. Uninfected LTA's gradually increased in cell number whereas VSV_{MS} infected LTA's decreased. Again, LTA's infected with Ad5 wt or AdG12 first increased in number and then decreased by 36 hours, although not as much as LTA's infected with VSV_{MS}.

Therefore, it appears that murine adherent cell lines B10.D2 and LTA were infectible with both Ad5 wt and AdG12 at an MOI of 15, since these cell lines showed decreased viability when incubated with either virus. Similar results occurred using adherent PAK ($H\text{-}2^b$) cells (not shown). In contrast, suspension cell lines P815 and EL-4 incubated with Ad5 wt or Adg12 did not show decreased viability. However, from these studies, it was difficult to conclude that these cells were not infected with adenovirus.

In order to further determine whether various murine cell lines were infectible with the adenovirus recombinant AdG12, the expression of viral proteins following infection was examined.

Figure 2: Viability of EL-4 ($H\text{-}2^b$) Cells Upon Virus Infection

The $H\text{-}2^b$ suspended cell line EL-4 was tested for infectibility with various virus isolates in the following manner. Groups of approximately 5×10^6 EL-4 cells were incubated alone (○) or with either VSV_{ms} at an MOI of 5 (●), wildtype Ad5 at an MOI of 15 (△) or with the adenovirus recombinant AdG12 at an MOI of 15 (▲). These populations were each incubated at 37°C for 36 hours. During this incubation, cells were counted approximately every 6 hours to determine the number that survived in the presence or absence of virus. Using trypan blue dye, it was possible to determine if cells were subjected to CPE due to virus infection. Results shown on this graph are an average of 3 experiments ± standard deviation (illustrated by error bars).

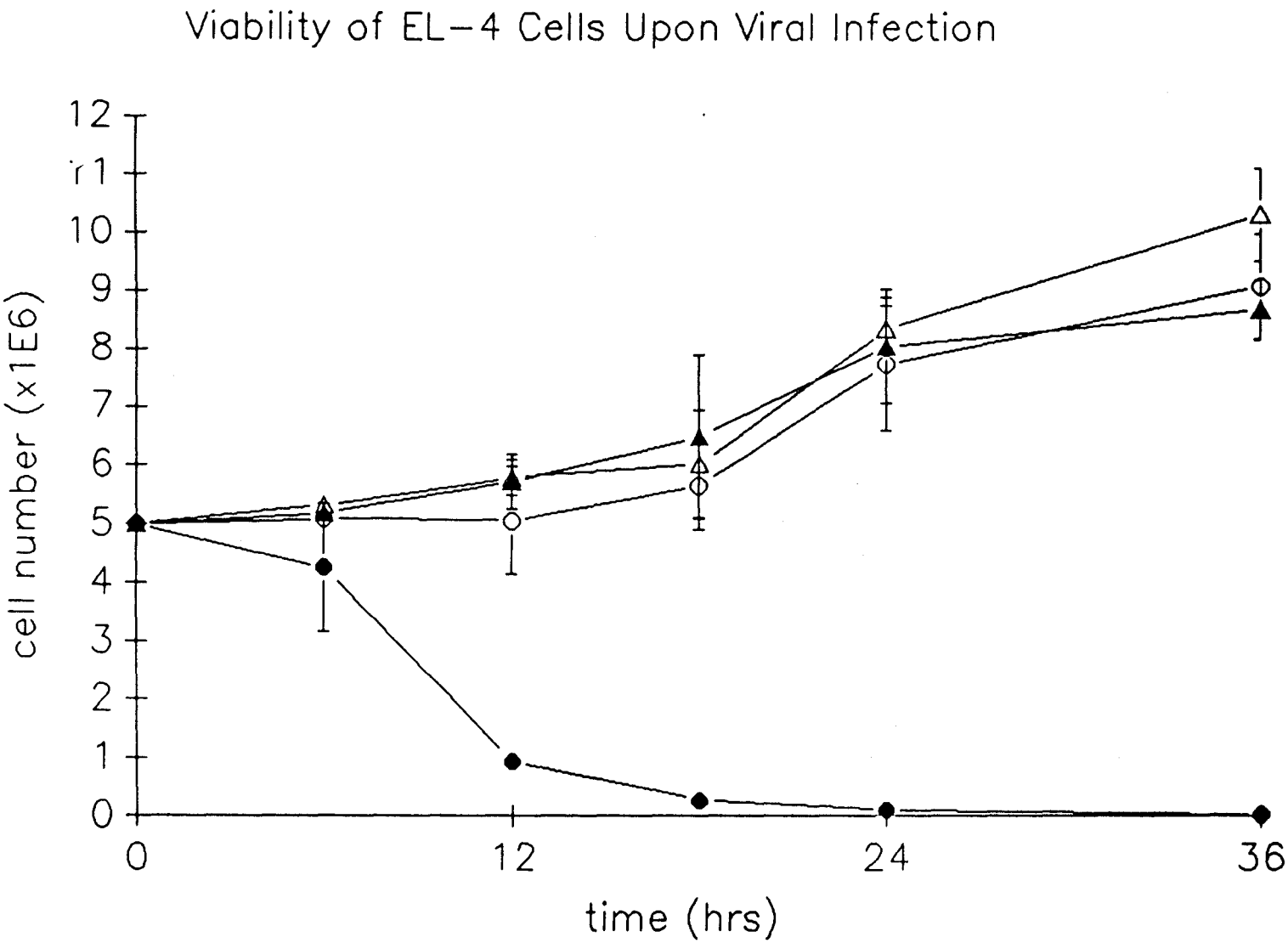

Viability of EL-4 Cells Upon Viral Infection
cell number (x1E6)
12
11
10
9
8
7
6
5
4
3
2
1
0
0
12
24
36
time (hrs)

Figure 3: Viability of B10.D2 ($H\text{-}2^d$) Cells Upon Virus Infection

The $H\text{-}2^d$ adherent cell line B10.D2 was tested for infectibility with various virus isolates in the following manner. Groups of approximately 5×10^6 B10.D2 cells were incubated alone (○) or with either VSV_{ms} at an MOI of 5 (●), wildtype Ad5 at an MOI of 15 (△) or with the adenovirus recombinant AdG12 at an MOI of 15 (▲). These populations were each incubated at 37°C for 36 hours. During this incubation, cells were counted approximately every 6 hours to determine the number that survived in the presence or absence of virus. Using trypan blue dye, it was possible to determine if cells were subjected to CPE due to virus infection. Results shown on this graph are an average of 3 experiments ± standard deviation (illustrated by error bars).

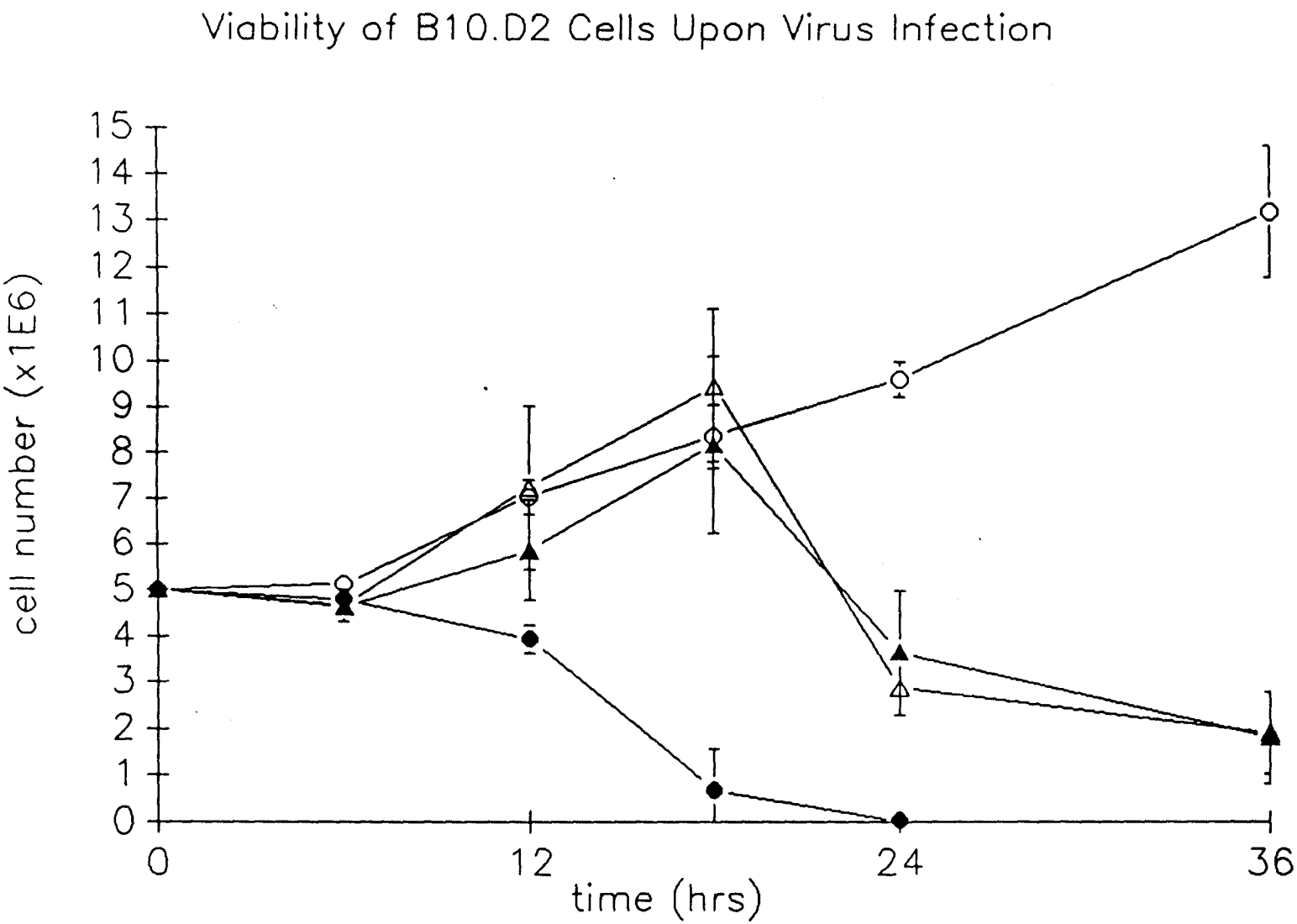
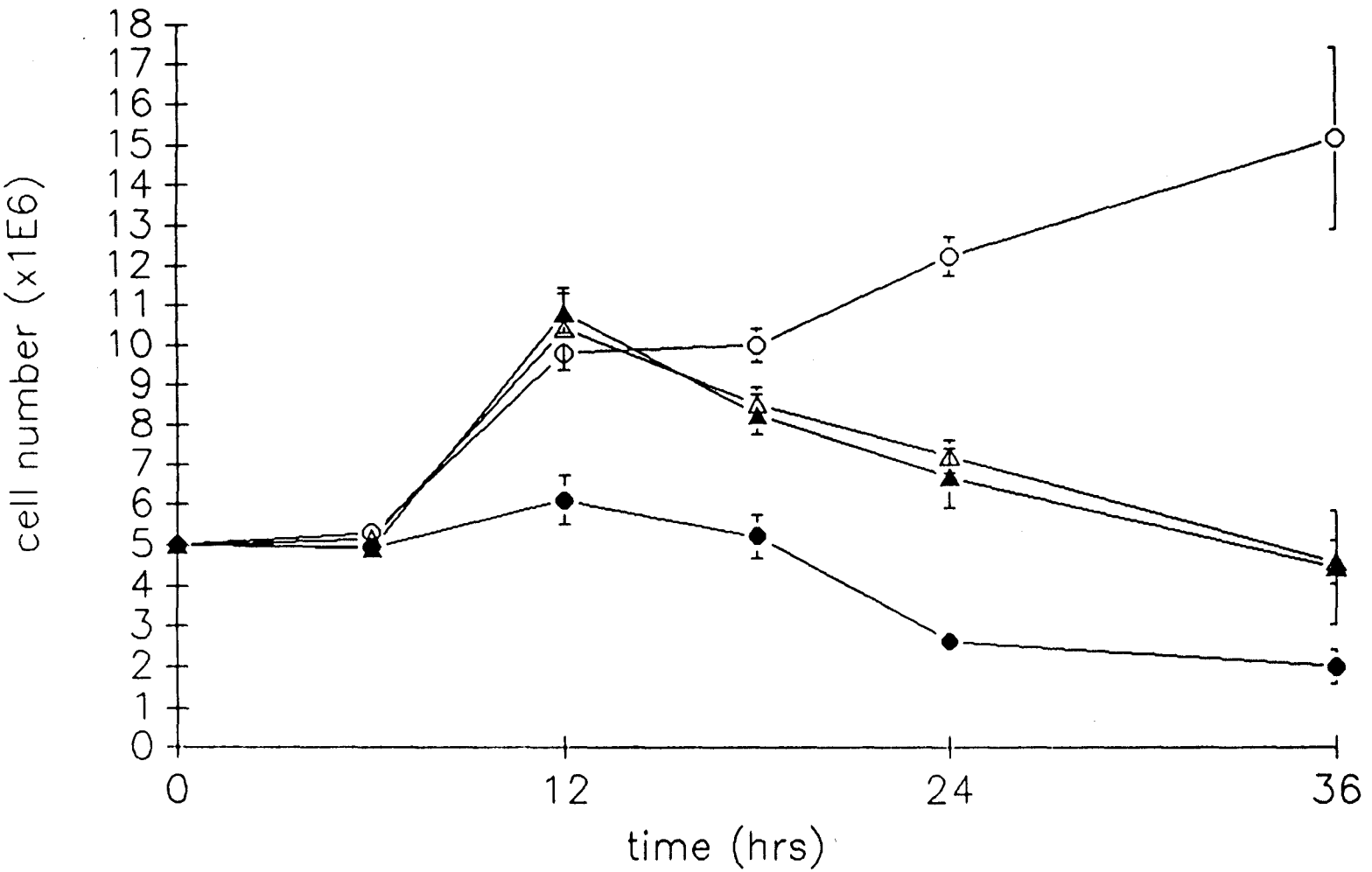

Viability of B10.D2 Cells Upon Virus Infection
cell number (x1E6)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
12
24
36
time (hrs)

Figure 4: Viability of LTA ($H\text{-}2^k$) Cells Upon Virus Infection

The $H\text{-}2^k$ adherent cell line LTA was tested for infectibility with various virus isolates in the following manner. Groups of approximately 5×10^6 LTA cells were incubated alone (O) or with either VSV_{ms} at an MOI of 5 (●), wildtype Ad5 at an MOI of 15 (Δ) or with the adenovirus recombinant AdG12 at an MOI of 15 (▲). These populations were each incubated at 37°C for 36 hours. During this incubation, cells were counted approximately every 6 hours to determine the number that survived in the presence or absence of virus. Using trypan blue dye, it was possible to determine if cells were subjected to CPE due to virus infection. Results shown on this graph are an average of 3 experiments ± standard deviation (illustrated by error bars).


Viability of LTA Cells Upon Virus Infection
cell number (x1E6)
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
12
24
36
time (hrs)

C/1.2 Expression of Viral Proteins Following Infection

Suspended and adherent murine cell lines were incubated with either VSV Indiana (either the Mudd Summers (MS) or the San Juan (SJ) strain), Ad5 wt or AdG12 recombinant for up to 48 hours. Each cell population was then analysed using SDS-polyacrylamide gel electrophoresis to measure the expression of VSV glycoprotein G. After 5 hours of incubation alone or with VSV (MS or SJ), cells were labelled with [^{35}S]-methionine for another hour. After labelling, they were washed and lysed. Cells infected with either Ad5 wt or AdG12 were labelled at various times post infection for one hour, washed and lysed. These individual cell extracts were then immunoprecipitated with monoclonal antibody 60-4, which specifically precipitates VSV-G and a polyspecific anti-VSV antibody used to enhance precipitation of the glycoprotein. In addition, measurement of Ad5 DNA binding protein (72K) was performed in order to determine whether murine cell lines could be infected with adenovirus (Ad5 wt or AdG12). Furthermore, these results indicated the time period of infection necessary for expression of these proteins.

Figure 5 represents SDS-PAGE data using the PAK (H-2^b) adherent cell line. Approximately 5 x 10^6 PAK cells were either uninfected or infected with VSV Indiana (MS or SJ) for 6 hours at an MOI of 5. As indicated, PAK cells were also

infected with AdG12 or Ad5 wt at an MOI of 15 for 6 to 36 hours.

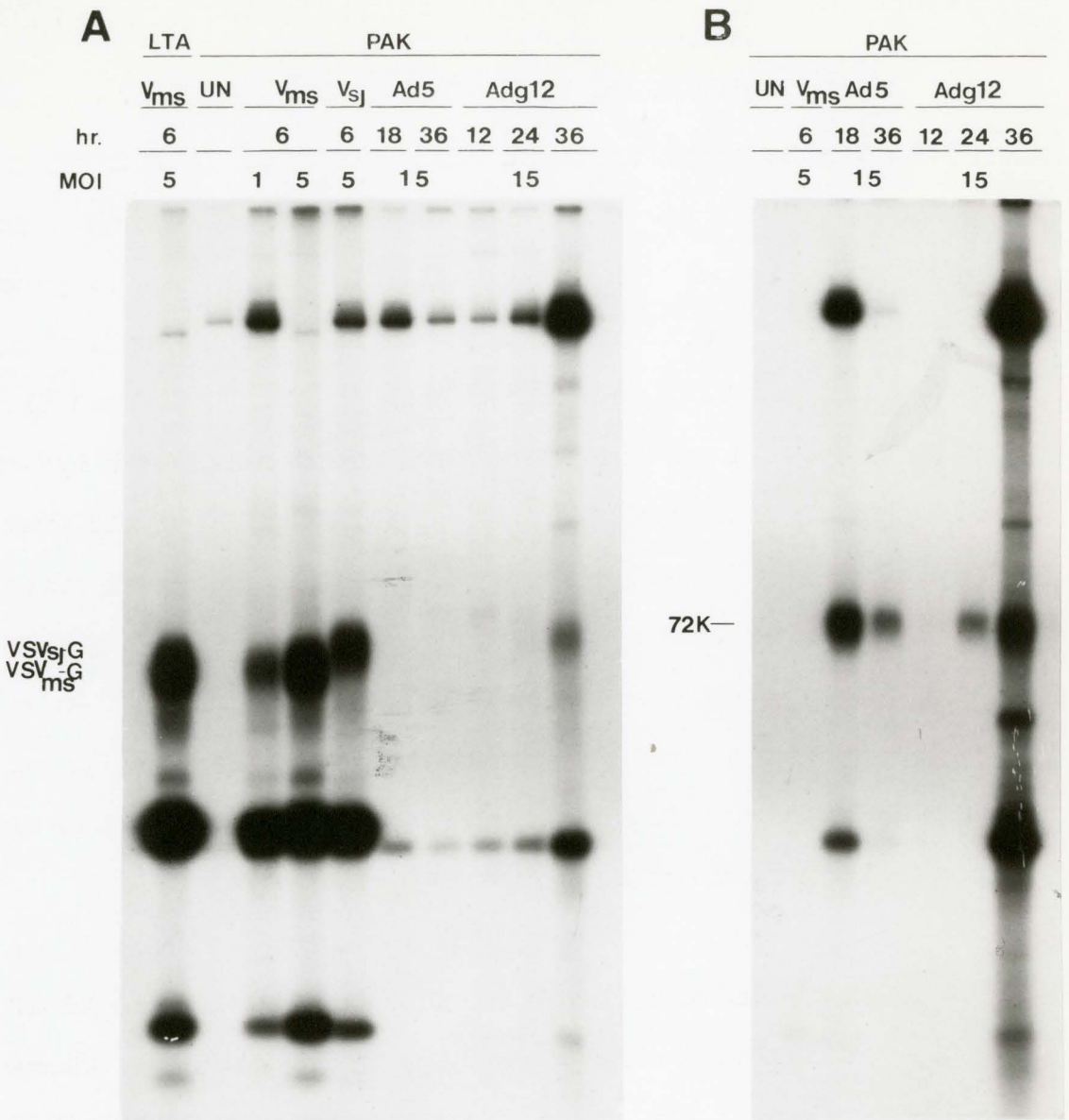
Results in figure 5A show that PAK cells infected with AdG12 expressed VSV-G (SJ) after 36 hours of infection (although no VSV protein was expressed at 12 and 24 hours) (lanes 8, 9 and 10). PAK cells infected with either VSV_{MS} or VSV_{SJ} showed expression of VSV-G as expected, but the San Juan protein ran at a slightly slower mobility than $VSV\text{-}G_{MS}$ (lanes 3, 4 and 5). As another positive control, the $H\text{-}2^k$ cell line LTA infected with VSV_{MS} for 6 hours, also expressed VSV-G (lane 1). Negative controls included uninfected PAK cells and those infected with Ad5 wt. These groups did not express VSV-G, even after 36 hours of infection (lanes 2, 6 and 7).

Figure 5B illustrates a similar experiment where the cell extracts were incubated with the monoclonal antibody H2-19 to immunoprecipitate the Ad5 DNA binding protein 72K. Results show that although uninfected and VSV_{MS} infected PAK cells did not express 72K (lanes 1 and 2), PAK cells infected with Ad5 (at an MOI of 15) did express 72K by 18 hours post-infection (lanes 3 and 4). Furthermore, PAK cells infected with AdG12 expressed 72K at 24 and 36 hours post-infection (lanes 5, 6 and 7).

Figure 5: Expression of $VSV\text{-}G_{SJ}$ in AdG12 Infected PAK Cells

A. PAK ($H\text{-}2^b$) cells grown to sub-confluency on 60mm petri dishes were infected with AdG12 at an MOI of 15. Infected cells were labelled with [^{35}S]-methionine for one hour, washed and lysed at 12, 24 and 36 hours post-infection. Control populations of PAK and LTA cells were infected with VSV_{MS} or VSV_{SJ} at an MOI of 5, labelled from 5 to 6 hours post-infection and then washed and lysed. Also cells infected with Ad5 at an MOI of 15 were labelled and lysed at 18 and 36 hours post-infection. These cell extracts along with those of uninfected PAK cells were incubated with monoclonal antibody 60-4 and a polyspecific anti-VSV antiserum to immunoprecipitate VSV-G protein. Precipitated proteins were eluted and electrophoresed on 10% polyacrylamide gels.

B. PAK cells were infected with VSV_{MS}, Ad5 or AdG12, [^{35}S]-methionine labelled and lysed as described above. Cell extracts were incubated with monoclonal antibody H2-19 to immunoprecipitate adenovirus protein 72K, prior to electrophoresis on 10% polyacrylamide gels.


A
LTA
PAK
Vms
UN
Vms
Vsj
Ad5
Adg12
hr.
6
6
6
18
36
12
24
36
MOI
5
1
5
5
15
15
VSVsjG
VSVmsG
B
PAK
UN
Vms
Ad5
Adg12
6
18
36
12
24
36
5
15
15
72K—

Therefore, in support of the viability studies previously performed, SDS-PAGE results in figure 5 show that the H-2^b PAK cell line is infectible with both Ad5 wt and the adenovirus recombinant AdG12. Furthermore, when infected with AdG12, PAK cells express VSV-G_{SJ} 36 hours post-infection.

SDS-PAGE data shown in figure 6 represents the same expression analysis on the adherent H-2^d cell line B10.D2. Figure 6B illustrates analysis of the B10.D2 cell extracts immunoprecipitated with anti-VSV-G monoclonal 60-4 and the polyspecific anti-VSV antibody. As seen, B10.D2 cells infected with AdG12 for 24, 36 or 48 hours all showed expression of VSV-G_{SJ} (not shown). Like PAK cells, B10.D2's infected with either VSV_{MS} or VSV_{SJ} for 6 hours at an MOI of 5 expressed VSV-G, in contrast to uninfected cells.

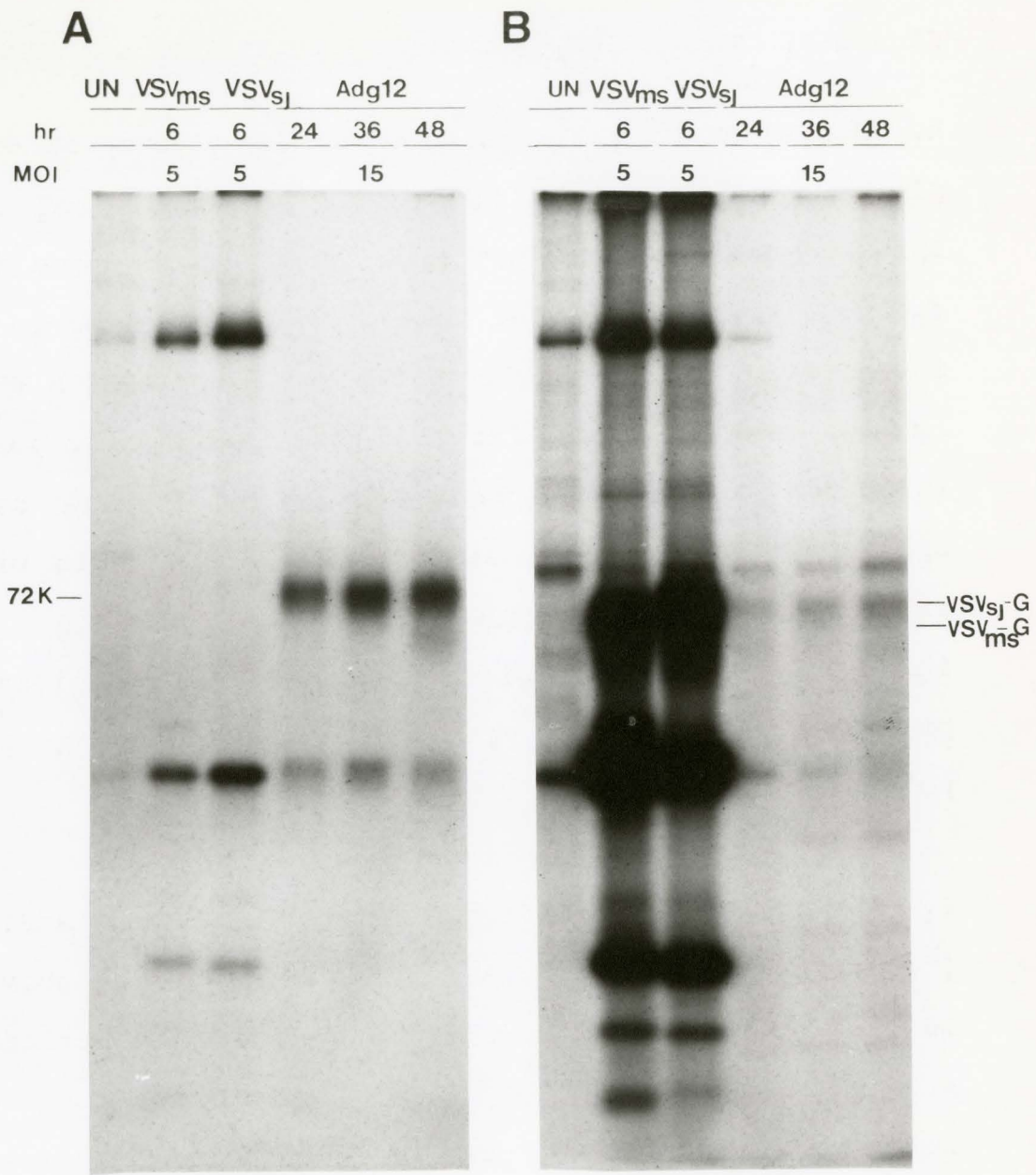
Figure 6A represents SDS-PAGE analysis of Ad5 72K expression in these B10.D2 cell extracts. As expected, only B10.D2 cells infected with AdG12 showed expression of 72K at 24, 36 and 48 hours post-infection.

Therefore the adherent cell line B10.D2 was also shown to be infectible with the AdG12 recombinant (confirming viability studies) and expressed VSV-G_{SJ} protein at least 24 hours post-infection.

Figure 6: Expression of $VSV\text{-}G_{SJ}$ in AdG12 infected B10.D2 Cells

A. B10.D2 ($H\text{-}2^d$) cells grown to sub-confluency on 60mm dishes were infected with AdG12 at an MOI of 15. Infected cells were labelled with [^{35}S]-methionine for one hour washed and lysed at 24, 36 and 48 hours post-infection. Control populations were infected with VSV_{MS} or VSV_{SJ} at an MOI of 5, labelled from 5 to 6 hours post-infection and then washed and lysed. These cell extracts, along with those from uninfected cells were incubated with monoclonal antibody H2-19 to immunoprecipitate adenovirus protein 72K. Precipitated proteins were eluted and electrophoresed on 10% polyacrylamide gels.

B. B10.D2 cells were infected with VSV_{MS}, VSV_{SJ} or AdG12, [^{35}S]-methionine labelled and lysed as described above. Cell extracts were then incubated with monoclonal antibody 60-4 and a polyspecific anti-VSV antiserum to immunoprecipitate VSV-G, prior to electrophoresis on 10% polyacrylamide gels.


A
B
UN
VSVms
VSVSJ
Adg12
hr
6
6
24
36
48
MOI
5
5
15
72K
VSVSJ-G
VSVms-G

As previously noted, both P815 (H-2^d) and EL-4 (H-2^b) suspended murine lines did not show decreased viability when incubated with adenoviruses Ad5 or AdG12. Thus, it was necessary to confirm whether these suspended lines were infected with adenovirus and could express viral specific proteins. SDS-PAGE analysis was therefore performed using the P815 cell line and results are displayed in figure 7.

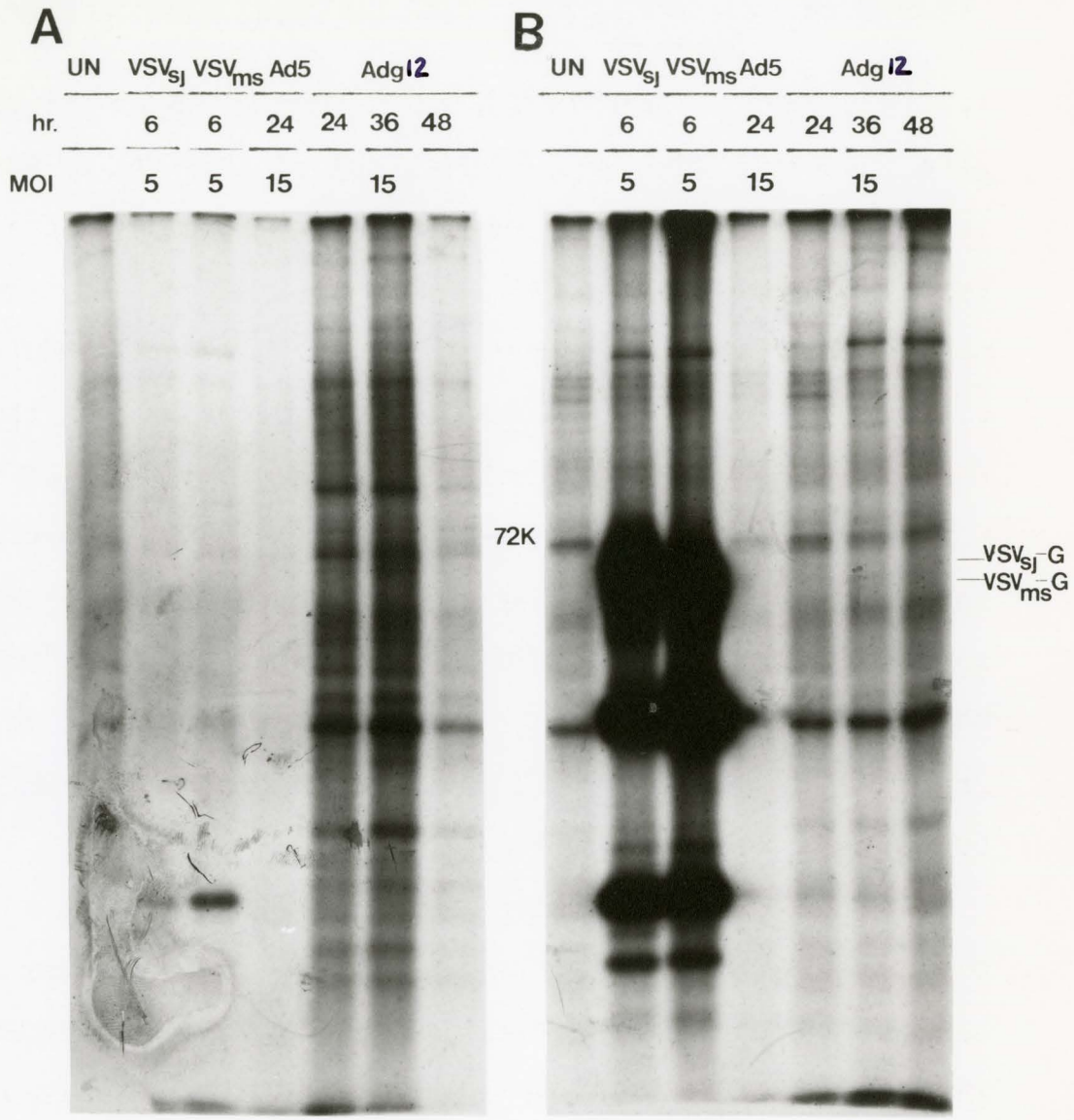
In figure 7A, uninfected P815 cells or those incubated with VSV (MS or SJ), Ad5 wt or AdG12 were analysed after immunoprecipitation with anti-72K antibody H2-19. Cells incubated with either Ad5 or AdG12 for 24, 36 and 48 hours showed no detectible expression of 72K. Uninfected and VSV infected P815's also did not express 72K, as expected.

Figure 7B shows the same cell extracts immunoprecipitated with anti-VSV-G monoclonal antibody 60-4 and the polyspecific anti-VSV antibody. Only P815 cells infected with VSV Indiana (MS or SJ) expressed strain specific VSV-G as seen on this gel. P815 cells infected with AdG12 for 24, 36 or 48 hours did not show any expression of VSV-G by this analysis. Therefore, P815 cells did not demonstrate detectable levels of either adenovirus specific 72K or VSV-G following infection with AdG12. By this analysis, it appeared that suspended cell lines such as P815 were not infectible with AdG12.

Figure 7: Expression of $VSV\text{-}G_{SJ}$ in AdG12 Infected P815 Cells

A. P815 ($H\text{-}2^d$) grown to subconfluency in 25 cm^2 flasks were incubated with AdG12 at an MOI of 15. Cells were labelled with [^{35}S]-methionine for one hour, washed and lysed after 24, 36 and 48 hours of incubation. Control populations were infected with VSV_{MS} or VSV_{SJ} at an MOI of 5, labelled from 5 to 6 hours post-infection and then washed and lysed. Also cells incubated with Ad5 at an MOI of 15 were labelled and lysed after 24 hours of incubation. These cell extracts along with those from uninfected cells were incubated with monoclonal antibody H2-19 to immunoprecipitate adenovirus protein 72K. Precipitated proteins were eluted and electrophoresed on 10% polyacrylamide gels.

B. P815 cells were infected with VSV_{MS}, VSV_{SJ}, Ad5 or AdG12, [^{35}S]-methionine labelled and lysed as described above. Cell extracts were then incubated with monoclonal antibody 60-4 and a polyspecific anti-VSV anti-serum to immunoprecipitate VSV-G, prior to electrophoresis on 10% polyacrylamide gels.


A
UN
VSV$_{sj}$
VSV$_{ms}$
Ad5
Adg12
hr.
6
6
24
24
36
48
MOI
5
5
15
15
B
UN
VSV$_{sj}$
VSV$_{ms}$
Ad5
Adg12
6
6
24
24
36
48
5
5
15
15
72K
VSV$_{sj}$-G
VSV$_{ms}$-G

C/2 Primary Murine CTL Responses Against AdG12

The cell-mediated immune response, particularly that of CTL activity, against VSV has been extensively studied (Zinkernagel et al., 1978a). A previous study by Rosenthal and Zinkernagel (1981) showed that although H-2^b and H-2^d mice mounted demonstrable CTL responses against VSV, H-2^k did not respond. Therefore Balb/c (H-2^d) and C57Bl/6 (H-2^b) mice were responders, whereas CBA/J (H-2^k) mice were not. It was also previously established that the primary cell mediated immune response against VSV Indiana peaks at about 6 days post intravenous infection of either Balb/c (H-2^d) or C57Bl/6 (H-2^b) mice (Zinkernagel et al., 1978a). Therefore in a ^{51}Cr release assay, target cells of either haplotype, infected with VSV Indiana, were killed to the highest degree by splenocyte effectors removed from mice 6 days after inoculation with virus.

In order to study the primary CTL response against the AdG12 recombinant virus, it was first necessary to establish when peak activity against this virus occurred in both Balb/c and C57Bl/6 mice.

C/2.1 Time Course of Primary CTL Response Against AdG12

Balb/c and C57Bl/6 mice were infected intravenously with 2.0×10^7 pfu of AdG12. The mice were then sacrificed six,

seven, eight and nine days after infection, at which time, their spleens were removed. These splenocytes were tested as effectors in a standard ^{51}Cr release assay against syngeneic target cells.

Data in table 1 represents ^{51}Cr release assays used to measure the response of Balb/c splenocyte effectors against B10.D2 uninfected and infected targets. Several conclusions can be made. Effectors from AdG12 infected mice were tested against uninfected, VSV, AdG12 or Ad5 wt infected targets. Those effectors removed from mice six days after AdG12 infection showed peaked responses against Ad5 wt and AdG12 infected B10.D2's. Furthermore, they also responded against VSV_{MS} and VSV_{SJ} infected targets. In fact this recognition of VSV targets was only marginally detected using day 7, 8 or 9 splenocytes. As a control, Balb/c spleen cells removed from mice 6 days after VSV_{MS} or VSV_{SJ} specific effectors responded against B10.D2 targets infected with AdG12 or Ad5 wt. However, both effector groups showed significant responses against B10.D2's infected with either VSV_{MS} or VSV_{SJ}. These results demonstrate that these two strains of VSV Indiana appear to be cross-reactively recognised.

TABLE 1

TIME COURSE OF PRIMARY CTL RESPONSE IN BALB/c MICE[a]

Balb/c Effectors[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] B10.D2 Targets				
		Uninf.	VSV-MS	VSV-SJ	Adg12	Ad5 wt.
Day 9 Adg12	40:1	7	13	14	46	45
	12:1	2	5	7	31	27
	4:1	3	2	2	22	21
Day 8 Adg12	40:1	6	14	14	46	50
	12:1	3	8	8	33	39
	4:1	2	5	3	19	28
Day 7 Adg12	40:1	4	16	13	50	57
	12:1	2	11	2	41	46
	4:1	2	7	<1	34	41
Day 6 Adg12	40:1	5	31	25	59	66
	12:1	5	20	15	50	55
	4:1	1	9	6	38	45
Day 6 VSV-MS	40:1	7	62	50	11	19
	12:1	3	46	43	5	11
	4:1	2	31	39	<1	2
Day 6 VSV-SJ	40:1	8	64	44	10	16
	12:1	5	51	35	7	9
	4:1	3	31	23	<1	5

[a]Results represent 1 of 3 experiments performed in duplicate.

[b]Balb/c mice were infected intravenously with VSV-MS, VSV-SJ, or Adg12 all at 2×10^7 pfu. Spleen cells were then removed 6 days after VSV infection of mice, and 6, 7, 8, or 9 days after Adg12 infection. Cytoxicity of spleen cells was tested against syngeneic B10.D2 targets as indicated.

[c]Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

Therefore, in the Balb/c H-2^d system, the activity of AdG12 specific spleen cell effectors against VSV, AdG12 and Ad5 wt infected targets peaked 6 days after inoculation of mice. It was thus considered the optimal time to measure CTL responses from Balb/c mice infected with either VSV or AdG12.

Table 2 represents similar ^{51}Cr release assays measuring C57Bl/6 effector responses against PAK cell targets. Once again, splenocytes taken from C57Bl/6 mice infected with AdG12 six days previously showed peaked responses against PAK targets infected with AdG12 and Ad5 wt. Also, recognition of VSV_{MS} or VSV_{SJ} infected targets by these AdG12 specific effectors was greater with day 6 than with day 7, 8 or 9 effector groups. Control VSV specific effector groups in the H-2^b system again showed a cross-reactive recognition of VSV_{MS} and VSV_{SJ} infected targets.

Therefore, by these results, day 6 was also the optimal time following infection of C57Bl/6 mice with AdG12 to measure CTL responses against VSV, AdG12 or Ad5 wt infected PAK targets.

With both the Balb/c and C57Bl/6 mice, AdG12 specific effectors mounted a demonstrable response against targets infected with VSV_{MS} or VSV_{SJ}. However, VSV specific effectors only recognised AdG12 infected targets to a minor

TABLE 2

TIME COURSE OF PRIMARY CTL RESPONSE IN C57Bl/6 MICE[a]

C57Bl/6 Effectors[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] PAK Targets				
		Uninf.	VSV-MS	VSV-SJ	Adg12	Ad5 wt.
Day 9 Adg12	40:1	2	6	5	14	28
	12:1	<1	5	2	7	15
	4:1	<1	<1	<1	5	8
Day 8 Adg12	40:1	5	8	4	16	29
	12:1	3	6	2	13	24
	4:1	<1	2	<1	6	14
Day 7 Adg12	40:1	1	10	5	27	35
	12:1	<1	6	<1	18	25
	4:1	<1	3	<1	8	18
Day 6 Adg12	40:1	5	13	15	29	39
	12:1	<1	7	9	18	28
	4:1	<1	5	4	9	20
Day 6 VSV-MS	40:1	4	46	37	16	22
	12:1	2	33	24	8	8
	4:1	<1	18	11	3	5
Day 6 VSV-SJ	40:1	4	36	27	8	19
	12:1	3	26	14	3	10
	4:1	<1	19	10	2	4

[a] Results represent 1 of 3 experiments performed in duplicate.

[b] C57Bl/6 mice were infected intravenously with VSV-MS, VSV-SJ or Adg12 all at 2 x 10^7 pfu. Spleen cells were then removed 6 days after VSV infection of mice, and 6, 7, 8, or 9 days after Adg12 infection. Cytoxicity of spleen cells was tested against syngeneic PAK targets as indicated.

[c] Assay duration was 6 hours at 37^0C. Spontaneous release was less than 30% for all targets.

degree in either mouse system. This recognition was no greater than that of Ad5 wt infected targets.

C/2.2 Cross-Reactivity Between VSV Indiana Strains

The adenovirus recombinant AdG12, as mentioned, contains the coding sequence for the G protein of the San Juan strain of VSV Indiana. Previous studies measuring the primary CTL response against VSV in mice used the Mudd Summers strain. Since results in tables 1 and 2 showed that VSV_{MS} and VSV_{SJ} were cross-reactively recognised, it was important to confirm this recognition. Thus, several ^{51}Cr release experiments were performed to measure this CTL response in Balb/c and C57Bl/6 mice.

Table 3 represents ^{51}Cr release data for the $H\text{-}2^{d}$ system. Splenocytes were taken from Balb/c mice 6 days after they were inoculated with VSV_{MS}, VSV_{SJ} or AdG12, and responses were measured against B10.D2 targets. As noted, both VSV_{MS} and VSV_{SJ} specific effectors responded against AdG12 and Ad5 wt infected targets to some degree more than against uninfected targets. More significantly, these Balb/c VSV specific spleen cells recognised both VSV_{MS} and VSV_{SJ} infected B10.D2 targets. In fact, effectors specific for either VSV strain responded against VSV_{MS} infected targets more than against VSV_{SJ} infected targets. This pattern of recognition was also seen using

AdG12 specific effectors. It is possible that VSV_{MS} infected targets expressed more VSV protein than VSV_{SJ} targets, accounting for the difference in recognition, but this was not confirmed. Nevertheless, VSV_{MS} and VSV_{SJ} specific Balb/c splenocytes did cross-reactively recognise VSV_{MS} and VSV_{SJ} infected B10.D2 targets in these experiments.

Table 4 demonstrates this pattern of cross-reactivity between VSV_{MS} and VSV_{SJ} in the $H\text{-}2^b$ system, measuring C57Bl/6 effectors against infected PAK cell targets.

Since it was noted that AdG12 specific effectors also showed slight recognition of VSV infected target cells, ^{51}Cr release assays were performed using effector cells from Balb/c mice inoculated six days previously with Ad5 wt. This additional effector control also showed this pattern against VSV_{MS} and VSV_{SJ} infected targets, supporting conclusions from tables 3 and 4. Results are displayed in table 5.

TABLE 3

PRIMARY CTL RESPONSES IN BALB/C MICE AGAINST TWO STRAINS OF VSV INDIANA[a]

Balb/c Effectors[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] B10.D2 Targets				
		Uninf.	VSV-MS	VSV-SJ	Adg12	Ad5 wt.
VSV-MS	40:1	7	70	59	15	20
	12:1	3	57	43	6	12
	4:1	2	44	34	3	6
VSV-SJ	40:1	4	76	56	10	16
	12:1	2	59	47	7	10
	4:1	<1	41	38	4	4
Adg12	40:1	7	25	20	67	81
	12:1	5	18	12	56	70
	4:1	<1	8	6	40	58

[a] Results represent 1 of 3 experiments performed in duplicate.

[b] Balb/c mice were infected intravenously with VSV-MS, VSV-SJ or Adg12 all at 2 x 10^7 pfu. Spleen cells were removed 6 days later and were assayed for cytotoxicity against B10.D2 targets as indicated.

[c] Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

TABLE 4

PRIMARY CTL RESPONSES IN C57Bl/6 MICE AGAINST TWO STRAINS OF VSV INDIANA[a]

C57Bl/6 Effectors[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] PAK Targets				
		Uninf.	VSV-MS	VSV-SJ	Adg12	Ad5 wt.
VSV-MS	40:1	4	43	33	10	15
	12:1	1	30	25	7	10
	4:1	2	16	16	4	6
VSV-SJ	40:1	2	40	27	9	15
	12:1	2	24	21	6	8
	4:1	<1	15	15	4	4
Adg12	40:1	3	14	14	34	35
	12:1	2	5	6	22	23
	4:1	<1	2	2	9	10

[a] Results represent 1 of 3 experiments performed in duplicate.

[b] C57Bl/6 mice were infected intravenously with VSV-MS, VSV-SJ or Adg12 all at 2 x 10^7 pfu. Spleen cells were removed 6 days later and were assayed for cytotoxicity against PAK targets as indicated.

[c] Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

TABLE 5

PRIMARY Ad5 wt SPECIFIC RESPONSE IN BALB/c MICE[a]

Balb/c Effectors[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] B10.D2 Targets				
		Uninf.	VSV-MS	VSV-SJ	Adg12	Ad5 wt.
VSV-MS	40:1	3	53	42	17	17
	12:1	1	37	26	7	8
	4:1	<1	22	17	2	3
VSV-SJ	40:1	3	51	39	11	13
	12:1	1	40	28	6	6
	4:1	1	22	21	4	5
Adg12	40:1	1	20	16	37	48
	12:1	1	14	9	26	36
	4:1	<1	8	4	20	26
Ad5 wt	40:1	3	15	7	48	57
	12:1	<1	8	5	34	47
	4:1	1	4	2	23	31

[a]Results represent 1 of 3 experiments performed in duplicate.

[b]Balb/c mice were infected intravenously with VSV-MS, VSV-SJ Adg12 or Ad5 wt all at 2 x 10^7 pfu. Spleen cells were removed 6 days later and were assayed for cytotoxicity against B10.D2 targets as indicated.

[c]Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

C/3. The Primary CTL Response Against Allogeneic Targets

As a control to validate the specificity of CTL responses with respect to MHC restriction, it was necessary to measure reactions of H-2^b and H-2^d effectors against infected allogeneic target cells. Table 6 represents ^{51}Cr release data using Balb/c effectors specific for VSV_{SJ} and AdG12. Firstly, VSV_{SJ} specific Balb/c effectors were tested against B10.D2 (syngeneic) targets infected with VSV_{SJ}, AdG12 and Ad5 wt. As previously shown, these effectors lysed VSV_{SJ} infected targets to a greater degree than uninfected cells, and they lysed AdG12 and Ad5 infected targets to a minor degree. Interestingly, these Balb/c effectors also lysed allogeneic PAK targets infected with VSV_{SJ}, although not as much as VSV_{SJ} infected syngeneic targets. Uninfected PAK cells and those infected with AdG12 and Ad5 were not lysed significantly. Secondly, AdG12 specific Balb/c effectors lysed B10.D2 targets in the established manner such that VSV_{SJ} infected B10.D2 cells were recognised, but not as much as AdG12 and Ad5 infected targets. Once again, only VSV_{SJ} infected allogeneic PAK cells were recognised by these AdG12 specific Balb/c effectors. Therefore, these two Balb/c effector groups showed very little or no MHC restriction with respect to recognition of VSV_{SJ}.

TABLE 6

PRIMARY CTL RESPONSES IN BALB/c MICE AGAINST ALLOGENEIC TARGETS[a]

Balb/c Effectors[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c]							
		B10.D2 Targets				PAK Targets			
		UN	VSV-SJ	Adg12	Ad5	UN	VSV-SJ	Adg12	Ad5
VSV-SJ	40:1	6	45	13	10	5	37	3	3
	12:1	3	37	10	7	4	22	<1	<1
	4:1	<1	16	2	2	<1	11	<1	<1
Adg12	40:1	5	24	41	44	6	19	6	5
	12:1	2	17	26	30	2	11	4	3
	4:1	<1	7	13	15	<1	5	2	1

[a]Results represent 1 of 3 experiments performed in duplicate.

[b]Balb/c mice were infected intravenously with VSV-SJ or Adg12 at 2 x 10^7 pfu. Spleen cells were removed 6 days later and were assayed for cytotoxicity against syngeneic B10.D2 and allogeneic PAK targets as indicated.

[c]Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

Responses against AdG12 and Ad5 however appeared to be $H\text{-}2^d$ restricted.

C/4. Control Experiments Using Splenocytes from Naive Mice

C/4.1 Splenocytes from Naive Balb/c Mice

Since virus specific Balb/c splenocytes were reacting in a positive fashion against VSV_{SJ} infected allogeneic targets, this measured killing appeared to be non-MHC restricted and therefore, non-CTL mediated. It was thus important to determine what type of killing activity was responsible for lysis of these VSV infected target cells. As a control to analyze this situation, spleen cells removed from Balb/c mice that had not been previously inoculated with virus were used as effector cells in the same ^{51}Cr release assays as described above. This would determine whether the killing activity was induced by virus infection of mice. In table 7, results compare the activity of these naive Balb/c effectors with effectors from mice inoculated 6 days previously with VSV_{SJ}, AdG12 of Ad5 wt. Again, release of ^{51}Cr from labelled syngeneic B10.D2 targets was used to measure activity from these effector groups. Along with the expected pattern of lysis seen with virus specific effectors, table 7 shows that naive splenocyte effectors significantly lysed VSV_{SJ} infected

targets. However, targets infected with AdG12 or Ad5 wt were only lysed to a very minor degree. Therefore, in the H-2^d system, VSV_{SJ} infection of B10.D2 targets seemed to make these targets sensitive even to splenocytes from uninfected Balb/c mice.

C/4.2 Splenocyte Effectors from Naive H-2^b, H-2^d and H-2^k Mice Against Syngeneic and Allogeneic Targets

Table 8 represents experiments using effector spleen cells from naive Balb/c, C57Bl/6 and CBA/J mice, measuring responses against a panel of uninfected and infected B10.D2, PAK and Z4(H-2^k) targets. Naive Balb/c splenocytes once again responded against VSV_{SJ} infected B10.D2 targets and negligibly against those infected with AdG12 or Ad5 wt. As another target control, B10.D2 cells were infected with the Ad5 derived mutant virus AddlE3, which lacks part of the non-essential E3 coding region. AddlE3 was a more suitable control than Ad5 wt since it contains only the adenovirus coding regions that are found in AdG12. Naive Balb/c effectors showed little response against these AddlE3 infected targets. Responses against allogeneic PAK targets were also measured and again, the Balb/c spleen cells significantly responded against VSV_{SJ} infected targets. However, when Z4 targets were used, these naive effectors did not respond against either uninfected or VSV_{SJ}, AdG12 or AddlE3 infected

target cells.

Results against this panel of targets using naive C57Bl/6 effectors are also displayed in table 8. These splenocytes mounted responses significantly against syngeneic PAK targets infected with VSV_{SJ} and against allogeneic VSV_{SJ} infected B10.D2 cells, as expected. Minor responses were also made against PAK and B10.D2 cells infected with either AdG12, Ad5 wt or Add1E3. Again, no detectable responses were made against any of the Z4 target groups.

Finally, CBA/J mice were used to obtain naive $H\text{-}2^k$ splenocyte effectors and their responses against these target cells were also measured. CBA/J mice were previously shown to be non-responders against VSV Indiana infected targets (Rosenthal and Zinkernagel, 1981). As expected, no response was made against Z4 targets. However, these naive CBA/J spleen cells did mount significant responses against both B10.D2 and PAK allogeneic targets infected with VSV_{SJ}.

Thus, it appeared that $H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}2^k$ naive splenocytes showed significant responses against both B10.D2 and PAK cell targets infected with VSV_{SJ} and to a very minor degree against these target cells infected with AdG12, Ad5 wt and Add1E3.

TABLE 7

PRIMARY CTL RESPONSES USING SPLEEN CELLS FROM NAIVE BALB/c MICE[a]

Balb/c Effectors[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] B10.D2 Targets			
		Uninf.	VSV-SJ	Adg12	Ad5 wt.
UNINF.	40:1	1	57	6	5
	12:1	<1	36	3	4
	4:1	<1	18	<1	1
VSV-SJ	40:1	9	42	20	18
	12:1	4	23	9	12
	4:1	2	14	8	4
Adg12	40:1	6	17	34	45
	12:1	3	10	20	31
	4:1	1	6	7	20
Ad5 wt	40:1	5	22	50	64
	12:1	3	11	31	50
	4:1	2	6	17	37

[a]Results represent 1 of 3 experiments performed in duplicate.

[b]Balb/c mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12 or Ad5 wt and spleen cells were removed 6 days later. These effector groups, along with spleen cells removed from uninfected mice, were assayed for cytotoxicity against B10.D2 targets as indicated.

[c]Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

TABLE 8

PRIMARY CTL RESPONSES USING NAIVE BALB/c, C57Bl/6 AND CBA/J SPLEEN CELLS[a]

Naive Spleens[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c]												
		B10.D2 Targets					PAK Targets				Z4 Targets			
		UN	VSV-SJ	Adg12	Ad5	AddlE3	UN	VSV-SJ	Adg12	AddlE3	UN	VSV-SJ	Adg12	AddlE3
BALB/c	40:1	2	43	8	6	11	6	26	11	10	3	3	1	3
	12:1	2	35	4	5	7	5	24	11	7	2	2	<1	1
	4:1	1	23	2	2	3	3	13	8	6	<1	<1	<1	<1
C57Bl/6	40:1	1	30	7	7	12	8	38	16	11	3	2	2	4
	12:1	<1	21	2	5	9	5	26	13	8	1	1	1	3
	4:1	<1	12	<1	<1	6	2	15	11	5	<1	1	<1	2
CBA/J	40:1	5	32	4	9	10	6	25	10	7	2	2	3	4
	12:1	3	20	3	6	7	4	19	6	4	<1	<1	<1	2
	4:1	<1	9	2	3	4	3	11	4	1	<1	<1	<1	1

[a]Results represent 1 of 3 experiments performed in duplicate.

[b]Spleen cells were removed from uninfected Balb/c, C57Bl/6 or CBA/J mice and were assayed for cytotoxicity against B10.D2, PAK and Z4 targets as indicated.

[c]Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

C/5 Naive and Virus Specific Effectors Against Syngeneic and Allogeneic Targets

For comparative purposes, responses using naive splenocytes against B10.D2, PAK and Z4 targets were performed in experiments using effectors from mice inoculated 6 days previously with VSV_{SJ}, AdG12, Ad5 wt or AddlE3.

Table 9 displays results using Balb/c effectors. Previously observed patterns of responses against each target group were demonstrated using naive and virus specific spleen cell effectors. Of interest was the fact that naive Balb/c splenocytes responded to a greater degree against VSV_{SJ} infected B10.D2 and PAK targets than did effectors from VSV_{SJ} infected mice.

Table 10 represents ^{51}Cr release assays measuring responses of C57Bl/6 effectors against syngeneic PAK targets and allogeneic Z4 and B10.D2 targets. Once again, responses of naive splenocytes against VSV_{SJ} infected PAK and B10.D2 cells were higher than those of VSV_{SJ} specific C57Bl/6 splenocytes. Again, no significant responses against Z4 and B10.D2 targets infected with AdG12, Ad5 wt or AddlE3 were made by any of the C57Bl/6 effector groups.

TABLE 9

NAIVE AND VIRUS SPECIFIC BALB/c EFFECTORS AGAINST SYNGENEIC AND ALLOGENEIC TARGETS[a]

Balb/c Eff.[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c]											
		B10.D2 Targets					PAK Targets					Z4 Targets	
		UN	VSV-SJ	Adg12	Ad5	AddlE3	UN	VSV-SJ	Adg12	Ad5	AddlE3	UN	VSV-SJ
UNINF.	40:1	4	68	8	5	12	5	30	12	10	7	3	4
	12:1	2	57	3	2	8	4	23	9	7	5	3	3
	4:1	2	32	2	<1	3	1	17	6	4	1	1	<1
VSV-SJ	40:1	4	52	16	10	18	8	26	11	12	12	2	6
	12:1	3	45	6	5	9	6	15	7	8	10	1	3
	4:1	1	24	4	1	4	4	7	5	5	6	<1	2
Adg12	40:1	4	28	38	44	35	7	19	13	12	9	3	5
	12:1	3	14	22	33	15	5	14	9	9	8	2	4
	4:1	<1	7	13	23	7	2	10	5	6	5	2	2
Ad5 wt	40:1	8	27	62	66	58	9	22	16	13	11	3	5
	12:1	3	16	46	56	41	8	10	7	7	9	3	3
	4:1	<1	5	31	42	21	<1	4	4	6	7	2	2
AddlE3	40:1	5	8	30	65	44	9	9	10	15	9	4	4
	12:1	3	2	19	57	31	4	5	6	12	7	2	3
	4:1	2	2	12	39	24	1	3	2	8	3	1	1

[a]Results represent 1 of 3 experiments performed in duplicate.

[b]Balb/c mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12, Ad5 wt or AddlE3 and spleen cells were removed 6 days later. These effector groups along with spleen cells removed from uninfected mice were assayed against B10.D2, PAK and Z4 targets as indicated.

[c]Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

TABLE 10

NAIVE AND VIRUS SPECIFIC C57Bl/6 EFFECTORS AGAINST SYNGENEIC AND ALLOGENEIC TARGETS[a]

C57Bl/6 Eff.[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c]											
		PAK Targets					B10.D2 Targets					Z4 Targets	
		UN	VSV-SJ	Adg12	Ad5	AddlE3	UN	VSV-SJ	Adg12	Ad5	AddlE3	UN	VSV-SJ
UNINF.	40:1	15	74	30	28	29	15	40	12	13	15	8	7
	12:1	7	58	23	16	17	9	28	4	7	7	6	3
	4:1	2	43	14	11	13	2	13	<1	4	5	3	1
VSV-SJ	40:1	8	66	19	12	13	9	23	3	6	8	8	9
	12:1	6	52	14	7	5	7	13	<1	3	5	4	6
	4:1	3	40	6	4	3	2	8	<1	1	3	2	2
Adg12	40:1	11	31	52	64	64	7	18	3	5	6		
	12:1	9	24	40	47	49	5	13	2	4	4	NT	NT
	4:1	4	14	28	32	34	2	7	<1	3	4		
Ad5 wt	40:1	13	36	65	70	71	5	19	6	7	9		
	12:1	7	27	52	58	62	3	11	2	4	7	NT	NT
	4:1	4	18	38	37	47	1	6	<1	2	3		
AddlE3	40:1	14	36	48	62	73	6	18	7	9	8		
	12:1	10	29	41	44	61	4	13	4	6	6	NT	NT
	4:1	6	14	30	27	53	1	5	1	4	2		

[a]Results represent 1 of 3 experiments performed in duplicate. NT = Not Tested.

[b]C57Bl/6 mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12, Ad5 wt or AddlE3 and spleen cells were removed 6 days later. These effector groups along with spleen cells removed from uninfected mice were assayed against B10.D2, PAK and Z4 targets as indicated.

[c] Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

Finally, naive and virus specific CBA effectors were tested in ^{51}Cr release assays against the B10.D2, PAK and Z4 targets. Results from table 11 show that naive CBA/J splenocytes responded highly against VSV_{SJ} infected B10.D2 cells and against VSV_{SJ} infected PAK cells, but only slightly against B10.D2's infected with AdG12 or AddlE3. The same pattern occurred using effectors from CBA/J mice intravenously injected (6 days previously) with VSV_{SJ}, AdG12 or AddlE3, such that all these effector groups recognised VSV_{SJ} infected B10.D2 and PAK targets. As expected, all CBA/J effector groups did not lyse syngeneic Z4 cells whether they were uninfected or infected with VSV_{SJ}, AdG12 or AddlE3.

Therefore, although these ^{51}Cr release assays were intended to measure specific cell-mediated (CTL) responses in mice, they seemed to demonstrate a non-specific, non-MHC restricted killing activity. It was thus necessary to determine what type of activity responsible for lysis of these infected targets was present in the $H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}2^k$ splenocyte effector populations.

TABLE 11

NAIVE AND VIRUS SPECIFIC CBA/J EFFECTORS AGAINST SYNGENEIC AND ALLOGENEIC TARGETS[a]

CBA/J Eff.[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c]									
		Z4 Targets				B10.D2 Targets				PAK Targets	
		UN	VSV-SJ	Adg12	AddlE3	UN	VSV-SJ	Adg12	AddlE3	UN	VSV-SJ
UNINF.	40:1	3	1	2	3	6	44	12	14	9	36
	12:1	4	1	2	2	5	30	7	11	7	23
	4:1	3	<1	<1	<1	3	18	5	7	5	16
VSV-SJ	40:1	4	3	2	2	9	35	11	14	9	29
	12:1	4	2	3	3	7	18	7	7	6	19
	4:1	3	2	2	2	1	10	4	4	4	13
Adg12	40:1	4	2	3	3	11	28	16	20	7	21
	12:1	4	2	3	2	7	17	10	13	4	16
	4:1	3	<1	1	1	5	10	5	9	2	7
AddlE3	40:1	5	1	4	5	9	34	15	18	6	15
	12:1	5	3	4	4	7	17	11	10	5	11
	4:1	4	1	2	2	4	12	6	6	3	6

[a] Results represent 1 of 3 experiments performed in duplicate.

[b] CBA/J mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12 or AddlE3 and spleen cells were removed 6 days later. These effector groups along with spleen cells removed from uninfected mice were assayed against Z4, B10.D2 and PAK targets as indicated.

[c] Assay duration was 6 hours at 37°C. Spontaneous release was less than 30% for all targets.

C/6. Primary Responses Against YAC-1 Targets Using Balb/c and C57Bl/6 Effectors

One attempt to define the killing activity in these effector populations was to perform the same ^{51}Cr release assays against YAC-1 target controls. The YAC-1 suspension cell line is typically used as a target cell to measure murine Natural Killer (NK) cell activity. Effector to YAC-1 target cell ratios at 100:1 have been previously used to sufficiently detect this activity.

Table 12 shows results measuring the response of naive and virus specific Balb/c spleen cell effectors against either uninfected YAC-1 cells or those infected with VSV_{SJ}. Each effector population was set up in the assay at a ratio of 100:1 and 25:1 with the YAC-1 targets. In comparison to the established responses against syngeneic (B10.D2) and allogeneic (PAK) target cells, it was clearly demonstrated that these Balb/c effectors did not significantly react against either uninfected YAC-1 targets or those infected with VSV_{SJ}. However, YAC-1 cells were not sufficiently tested for VSV infectibility. Table 13 represents the same set of experiments performed using naive and virus specific C57Bl/6 splenocytes. Again, these effectors showed no significant responses against uninfected and VSV_{SJ} infected YAC-1 targets at either the 100:1 or the 25:1 ratios, when compared to responses against PAK and B10.D2 targets.

TABLE 12

PRIMARY RESPONSES AGAINST YAC-1 TARGETS USING BALB/c EFFECTORS[a]

Balb/c Eff.[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] B10.D2 Targets				PAK Targets				E:T Ratio	YAC-1 Targets	
		UN	VSV-SJ	Adg12	AddlE3	UN	VSV-SJ	Adg12	AddlE3		UN	VSV-SJ
UNINF.	40:1	<1	35	6	9	4	22	7	7	100:1	4	6
	12:1	<1	19	4	6	3	11	5	5	25:1	2	5
	4:1	<1	14	2	3	<1	6	2	3			
VSV-SJ	40:1	<1	27	10	10	6	19	9	8	100:1	2	5
	12:1	<1	17	5	6	4	10	7	3	25:1	<1	3
	4:1	<1	7	<1	4	1	6	5	1			
Adg12	40:1	2	16	19	19	5	18	11	5	100:1	3	7
	12:1	<1	10	10	9	2	9	6	4	25:1	1	4
	4:1	<1	7	4	4	<1	4	3	2			
AddlE3	40:1	2	16	21	21	4	16	10	6	100:1	6	9
	12:1	<1	9	9	10	2	7	6	4	25:1	2	6
	4:1	<1	5	3	5	2	4	4	1			

[a] Results represent 1 of 3 experiments performed in duplicate.

[b] Balb/c mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12 or AddlE3 and spleen cells were removed 6 days later. These effector groups along with spleen cells removed from uninfected mice were assayed against B10.D2, PAK and YAC-1 targets. E:T ratios for YAC-1 targets were at 100:1 and 25:1 as indicated.

[c] Assay duration at 37°C was 4 hours for YAC-1 targets and 6 hours for all other targets. Spontaneous release was less than 30% for all targets.

TABLE 13

PRIMARY RESPONSES AGAINST YAC-1 TARGETS USING C57Bl/6 EFFECTORS[a]

C57Bl/6 Eff.[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] PAK Targets				B10.D2 Targets				E:T Ratio	YAC-1 Targets	
		UN	VSV-SJ	Adg12	AddlE3	UN	VSV-SJ	Adg12	AddlE3		UN	VSV-SJ
UNINF.	40:1	10	47	20	23	12	28	10	13	100:1	8	8
	12:1	7	35	12	19	7	20	7	8	25:1	5	4
	4:1	5	27	8	9	4	10	4	5			
VSV-SJ	40:1	6	41	14	15	6	21	5	6	100:1	3	5
	12:1	4	29	9	9	4	15	2	3	25:1	<1	3
	4:1	3	18	4	7	<1	7	2	2			
Adg12	40:1	7	20	34	44	8	17	3	7	100:1	<1	2
	12:1	5	12	26	28	5	8	2	4	25:1	<1	1
	4:1	3	7	16	20	2	4	<1	3			
AddlE3	40:1	6	19	29	45	4	15	4	6	100:1	<1	5
	12:1	3	12	20	30	3	12	2	3	25:1	<1	2
	4:1	2	6	11	21	3	7	2	2			

[a] Results represent 1 of 3 experiments performed in duplicate.

[b] C57Bl/6 mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12 or AddlE3 and spleen cells were removed 6 days later. These effector groups along with spleen cells removed from uninfected mice were assayed against B10.D2, PAK and YAC-1 targets. E:T ratios for YAC-1 targets were at 100:1 and 25:1 as indicated.

[c] Assay duration at 37°C was 4 hours for YAC-1 targets and 6 hours for all other targets. Spontaneous release was less than 30% for all targets.

Therefore, by analysing responses against YAC-1 targets, evidence of NK activity was not detected in either the Balb/c or C57Bl/6 effector populations. However, VSV_{SJ} infected B10.D2 and PAK targets were still lysed significantly by each effector group in a non-specific, non-MHC restricted manner, refuting evidence for CTL activity.

C/7. Primary Responses Using Adherent Cell Depleted Splenocyte Populations

The splenocyte populations derived from naive and virus inoculated mice were clearly crude extracts that include a variety of cell types. Therefore, this non-specific killing activity was likely due to a non-CTL cell type in these effector populations. In order to distinguish such killer cells, spleen cell extracts were treated, before analysis in ^{51}Cr release assays, to isolate particular cell types. In one attempt, Balb/c splenocytes were incubated on plastic petri dishes for one hour at 37°C, to remove only adherent cells. The remaining non-adherent cell types were then used as effectors against B10.D2, PAK and YAC-1 targets. Table 14 displays these reactions against targets uninfected or infected with VSV_{SJ}, AdG12 or AddlE3. Results were compared to those using untreated splenocyte populations against the same targets. It seemed evident that the pattern of responses between Balb/c effectors and each target cell at each ratio

was similar using crude spleen cell populations and those depleted of adherent cells. Percentage of ^{51}Cr release was negligibly lower using the treated effectors. Thus, it appeared that adherent cells (such as macrophages) were not responsible for the killing activity within these Balb/c splenocyte populations.

TABLE 14

PRIMARY RESPONSES USING ADHERENT CELL DEPLETED BALB/c SPLENOCYTE POPULATIONS[a]

Balb/c Eff.[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] B10.D2 Targets				PAK Targets				E:T Ratio	YAC-1 Targets	
		UN	VSV-SJ	Adg12	AddlE3	UN	VSV-SJ	Adg12	AddlE3		UN	VSV-SJ
UNINF.	40:1	1	60	7	7	10	31	13	14	100:1	5	6
	12:1	<1	43	4	4	6	20	8	7	25:1	NT	NT
	4:1	<1	35	1	2	2	8	4	4			
VSV-SJ	40:1	4	45	14	14	12	23	10	11	100:1	7	8
	12:1	2	29	9	8	5	15	6	5	25:1	NT	NT
	4:1	1	17	5	4	4	6	3	2			
Adg12	40:1	5	22	47	41	12	20	10	8	100:1	<1	1
	12:1	3	13	24	23	7	13	6	5	25:1	NT	NT
	4:1	2	8	16	14	2	8	3	3			
AddlE3	40:1	7	23	51	55	10	19	12	10	100:1	2	4
	12:1	3	11	36	42	4	10	6	6	25:1	NT	NT
	4:1	2	5	20	24	2	5	4	2			
UNINF. Adherent Cell Dep.	40:1	2	57	5	5	7	27	11	12	100:1	4	5
	12:1	<1	41	3	5	3	15	6	6	25:1	NT	NT
	4:1	<1	30	<1	2	2	8	4	3			
VSV-SJ Adherent Cell Dep.	40:1	4	41	11	11	10	20	8	9	100:1	7	8
	12:1	1	28	6	5	4	9	5	5	25:1	NT	NT
	4:1	<1	15	4	3	3	5	2	1			
Adg12 Adherent Cell Dep.	40:1	4	20	44	38	11	17	7	8	100:1	<1	<1
	12:1	3	12	22	20	6	11	6	4	25:1	NT	NT
	4:1	<1	6	16	13	3	6	1	1			
AddlE3 Adherent Cell Dep.	40:1	6	21	48	52	9	18	10	9	100:1	3	3
	12:1	4	13	30	40	4	11	6	4	25:1	NT	NT
	4:1	1	6	20	22	2	7	3	2			

[a] Results represent 1 of 3 experiments performed in duplicate. NT = Not Tested.

[b] Balb/c mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12 or AddlE3 and spleen cells were removed 6 days later. Along with naive Balb/c spleen cells, these effector groups were either untreated or treated to remove adherent cells. All spleen cell populations were then assayed for cytotoxicity against B10.D2, PAK and YAC-1 targets as indicated.

[c] Assay duration at 37°C was 4 hours for YAC-1 targets and 6 hours for all other targets. Spontaneous release was less than 30% for all targets.

C/8. Primary Responses Using T-Cell Depleted Splenocyte Populations

Further analysis of crude splenocyte extracts from these Balb/c mice involved the depletion of T cells. Splenocyte populations were treated with anti-Thy 1.2 monoclonal antibody and rabbit complement in order to remove existing T cells such as CTL's. Each spleen cell group (from naive or virus inoculated mice) was analysed by Fluorescence Activated Cell Sorting (FACS) to determine the ratio of T to B cells before and after the depletion process. Results in table 15 show the successful depletion of T cells in each case. Following this analysis, untreated and treated splenocytes were tested as effectors in ^{51}Cr release assays against B10.D2, PAK and YAC-1 targets. Table 16 outlines these results. It was clear that responses against each target were similar using effector populations with or without T cells. This was particularly demonstrated in the case of splenocyte effectors from unstimulated mice. However, some differences were evident. Treated splenocytes from VSV_{SJ} infected mice showed significantly lower responses against syngeneic targets infected with VSV_{SJ} than did untreated effectors. AdG12 infected targets were also not killed as much using depleted populations. Furthermore, both AdG12 and AddlE3 specific effector groups responded significantly less toward syngeneic targets infected with AdG12 or AddlE3 when depleted of T

cells. Responses against VSV_{SJ} infected targets remained unchanged with or without treatment of these effectors.

Therefore, results in this particular study demonstrated the following. Firstly, the non-specific killing activity found in naive and virus inoculated Balb/c splenocyte populations was not T cell derived. Also, some T cell derived, MHC restricted killing may have been evident in VSV_{SJ} specific effector groups. This was demonstrated against VSV_{SJ} infected targets. It is also possible that AdG12 infected targets were lysed specifically by these effectors. Splenocytes from AdG12 and AddlE3 infected mice only specifically lysed targets infected with these two adenoviruses.

TABLE 15

FACS ANALYSIS OF BALB/c SPLENOCYTE POPULATIONS[a]

Balb/c Effectors	PERCENT T CELLS	PERCENT B CELLS
UNINF.	45.7	46.0
VSV-SJ	49.4	43.2
Adg12	50.9	40.3
AddlE3	51.4	34.8
UNINF. T Cell Depleted	6.6	87.9
VSV-SJ T Cell Depleted	6.3	88.8
Adg12 T Cell Depleted	4.8	90.6
AddlE3 T Cell Depleted	6.1	91.4

[a]Balb/c mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12 or AddlE3 and spleen were removed 6 days later. Along with naive Balb/c spleen cells, these effector groups were either untreated or treated to remove Thy 1+ (T) cells. All spleen cell populations were then FACS analysed to determine the number of T versus B cells.

TABLE 16

PRIMARY RESPONSES USING T CELL DEPLETED BALB/c SPLENOCYTE POPULATIONS[a]

Balb/c Eff.[b]	E:T Ratio	Percent Specific ^{51}Cr Release[c] B10.D2 Targets UN	VSV-SJ	Adg12	AddlE3	PAK Targets UN	VSV-SJ	Adg12	AddlE3	E:T Ratio	YAC-1 Targets UN	VSV-SJ
UNINF.	40:1	3	66	10	14	7	37	14	13	100:1	5	7
	12:1	1	43	6	9	5	24	9	7	25:1	NT	NT
	4:1	<1	31	3	4	3	15	4	5			
VSV-SJ	40:1	4	56	21	22	5	33	13	11	100:1	4	7
	12:1	3	32	14	13	3	24	8	6	25:1	NT	NT
	4:1	<1	20	6	6	2	13	5	4			
Adg12	40:1	7	27	47	43	9	19	14	17	100:1	6	9
	12:1	4	19	24	22	5	11	8	9	25:1	NT	NT
	4:1	1	8	13	11	3	6	5	4			
AddlE3	40:1	8	29	52	49	7	17	14	13	100:1	5	9
	12:1	4	17	30	27	2	10	9	8	25:1	NT	NT
	4:1	2	8	18	15	2	5	6	3			
UNINF.	40:1	2	63	10	13	5	36	15	13	100:1	4	9
T Cell	12:1	1	47	5	8	4	20	6	7	25:1	NT	NT
Depleted	4:1	<1	34	1	4	2	14	4	4			
VSV-SJ	40:1	4	34	14	20	4	33	13	11	100:1	4	6
T Cell	12:1	2	15	6	10	2	22	7	6	25:1	NT	NT
Depleted	4:1	<1	6	3	6	1	9	4	3			
Adg12	40:1	6	26	24	23	9	20	14	15	100:1	4	7
T Cell	12:1	2	22	15	15	4	10	10	9	25:1	NT	NT
Depleted	4:1	1	10	7	4	3	5	5	5			
AddlE3	40:1	7	26	29	25	6	21	15	16	100:1	5	9
T Cell	12:1	3	17	17	14	4	13	7	5	25:1	NT	NT
Depleted	4:1	<1	14	6	4	2	5	3	5			

[a] Results represent 1 of 3 experiments performed in duplicate. NT = Not Tested.

[b] Balb/c mice were infected intravenously with 2 x 10^7 pfu of VSV-SJ, Adg12 or AddlE3 and spleen cells were removed 6 days later. Along with naive Balb/c spleen cells, these effector groups were either untreated or treated to remove Thy 1+ (T) cells. All spleen cell populations were then assayed for cytotoxicity against B10.D2, PAK and YAC-1 targets as indicated.

[c] Assay duration at 37°C was 4 hours for YAC-1 targets and 6 hours for all other targets. Spontaneous release was less than 30% for all targets.

D/. DISCUSSION

The present studies were undertaken to examine the specificity of murine cell-mediated immune responses using recombinant adenovirus vectors. The recombinant virus AdG12, which contains the coding region for VSV glycoprotein, provided a model system to study anti-VSV CTL responses against syngeneic target cells expressing VSV-G.

A number of murine cell lines were tested for their ability to be productively infected with AdG12. It was necessary to determine if and when infected cells expressed VSV-G so that they could be used as targets for VSV-specific killers. It appeared that of the cell lines examined, B10.D2 ($H\text{-}2^d$) and PAK ($H\text{-}2^b$) lines were infectible with AdG12 and expressed VSV-G by 36 hours post-infection (VSV-G was detected in B10.D2 cells 24 hours post-infection).

Cell-mediated immunity to AdG12 was examined using Balb/c ($H\text{-}2^d$) and C57Bl/6 ($H\text{-}2^b$) mice. These studies demonstrated that splenocyte effectors from mice primed six days previously with AdG12 lysed syngeneic target cells infected with AdG12 or Ad5 wt. AdG12 primed mice, therefore, appear to have produced CTL's specific for adenovirus proteins. Interestingly, AdG12 primed effectors also lysed VSV infected syngeneic targets to a minor extent. In contrast, spleen cells from VSV primed mice did not show significant lysis of AdG12 infected targets. It, therefore, appeared that VSV primed effectors did not recognize VSV-G on the surface of AdG12 infected targets. However, in the reciprocal situation,

AdG12 primed effectors recognized some moiety on the surface of VSV infected targets.

To examine the specificty of responses from AdG12 primed mice, several control experiments were performed. In this analysis, effectors from AdG12 primed mice showed demonstrable lysis of VSV infected allogeneic targets. VSV primed effectors also significantly lysed allotargets infected with VSV. Thus, it seemed that B10.D2 and PAK targets infected with VSV were recognized by AdG12 and VSV primed effectors in a non-MHC restricted manner.

Lack of MHC restriction implied that effectors from AdG12 and VSV primed mice did not kill VSV infected targets in a classical CTL manner. Further experiments attempted to show what type of cells or factors were responsible for this cytotoxic activity and, whether it was induced by VSV or AdG12 infection of these mice.

Experiments using splenocytes from uninfected mice were performed to determine if this cytotoxic activity was virus induced. Results demonstrated that spleen cells from uninfected Balb/c, C57Bl/6 and CBA/J ($H\text{-}2^k$) significantly lysed VSV infected B10.D2 and PAK targets. This indicated that cytotoxicity may not have been AdG12 or VSV induced. Instead, these particular mouse strains displayed an inherent cytotoxic activity to which VSV infected B10.D2 and PAK targets were susceptible.

Splenocyte effector populations from naive and virus

infected mice were further examined to characterize this cytotoxic activity. Depletion experiments demonstrated that this activity was not derived from adherent cells or Thy1 bearing (T) cells. The presence of non-CTL killers in these spleen cell extracts, that displayed natural cytotoxicity against virus infected cells, was also tested. Previously, lysis of YAC-1 targets was shown to indicate natural killer (NK) activity in murine spleens (Kiessling et al., 1975). However, in the present studies, Balb/c and C57Bl/6 mice did not show significant lysis of YAC-1 targets, implying that NK activity may not have been present.

The implications of these studies and directions for future endeavours are discussed below.

D/1. Expression of VSV-G Following AdG12 Infection

The productive cycle of infection by human adenoviruses has been previously determined using human cell lines HeLa and KB (Flint and Broker, 1980). There are essentially two phases to this cycle, early and late, and they are delineated by the onset of viral DNA replication (Green et al., 1971). In the early phase, a portion of the viral genome is expressed as mRNA, leading to the synthesis of a small number of viral proteins. Once viral DNA replication is initiated, the late phase begins, and the amount of mRNA greatly increases until approximately 18 hours post-infection (Lindberg et al., 1972). This late mRNA accounts for most of the viral genome

expression and directs the synthesis of large amounts of viral structural proteins (Green et al., 1971).

Synthesis of viral DNA begins about 6-8 hours after infection of human cells with Ad5, reaching a maximum approximately 6-10 hours later. By this time, host DNA synthesis and host protein replication have been shut off (Ginsberg et al., 1967). Thus, the early phase of infection is independent of viral DNA replication and occurs before inhibition of host protein synthesis; in contrast, late phase protein synthesis is viral DNA synthesis dependent.

The expression of foreign genes inserted into adenoviral vectors has been recently considered in light of these phase kinetics (Johnson et al., 1988; Morin et al., 1987; Davidson and Hassell, 1987). Schneider et al. (1989) studied VSV-G expression in human HeLa cells infected with AdG12. As mentioned, the AdG12 vector consists of a complete Ad5 genome, except for a deletion in the non-essential E3 coding region. The VSV-G gene, between an HSV-1 TK promoter and a TK poly A addition site, was inserted next to the E3 promoter, in an orientation parallel to the E3 transcription unit (Schneider et al., 1989). VSV-G was thought to be expressed from the Ad E3 promoter since more VSV-G transcripts were detected using the AdG12 vector than using AdG4, a recombinant vector where the VSV-G gene was inserted in the opposite orientation to the E3 transcription unit (Schneider et al., 1989). This was supported by studies where a promoterless gene for a hepatitis

B surface antigen was inserted in front of the Ad5 E3 promoter. Cells infected with this vector expressed large amounts of the hep B antigen (Morin et al., 1987).

HeLa cells infected with AdG12 were shown to express surface VSV-G protein 6-7 hours post-infection, during the early phase of Ad5 protein kinetics before viral DNA replication. To confirm this, infected cells were treated with the DNA replication inhibitor cytosine arabinoside (ara-C). Ara-C treated cells still expressed VSV-G, demonstrating that in HeLa cells infected with AdG12, VSV-G was expressed as an early Ad gene (Schneider et al., 1989).

Adenovirus infection and protein synthesis kinetics depend, to some extent, on the type of host cell (Flint and Broker, 1980; Ledinko, 1970). In contrast to studies using human HeLa cells, Tremblay et al. (1985) found that mouse cells were only semi-permissive for Ad5 replication and showed greatly retarded kinetics of expression. The murine cell line BS3 infected with Ad5 demonstrated an early phase of Ad replication 14-18 hours post-infection. At this time, host protein synthesis had not yet been inhibited. Furthermore, expression of the early phase protein 72K was not detected until 15-16 hours post-infection and reached maximum levels 18 hours post-infection (Tremblay et al., 1985).

Studies on the infection of mouse cells with AdG12 were consistent with these observations. Prevec et al. (1989) demonstrated that murine L cells infected with AdG12 did not

express VSV-G until 14-18 hours post-infection, which was still within the early phase of the Ad5 infection cycle in mice.

In the present studies, B10.D2 cells infected with AdG12 did not demonstrate detectible expression of VSV-G until 24 hours post-infection. VSV-G was detected 36 hours post AdG12 infection of PAK cells. Thus, in these AdG12 infected murine cell lines, early phase kinetics appeared to be even further retarded. To confirm this, B10.D2 and PAK cells infected with AdG12 were analysed for expression of the Ad5 early protein 72K. In both cell lines, 72K was not detected until 24 hours post AdG12 infection and was still present 12 hours later. This implied that VSV-G may have been expressed during the early phase of infection. However, further experiments to delineate the onset of Ad DNA replication in these infected cells should be performed. In this manner, the kinetics of a productive AdG12 infection of B10.D2 and PAK cells would be more conclusively determined.

D/2. H-2 Restriction of the Anti-VSV Response

Cytotoxic T lymphocytes (CTL) represent one type of virus specific cell-mediated immune response elicited following virus infection of a particular host (reviewed in Borysiewicz and Sissons, 1986). CTL's demonstrate virus antigen specificity and a restriction by gene products encoded in the major histocompatibility complex (MHC) (Zinkernagel and

Doherty, 1979). In the mouse, MHC is referred to as H-2 and is found on chromosome 17. Zinkernagel and Doherty (1979) previously showed that murine anti-viral CTL are generally restricted by the Class I H-2K and H-2D regions of MHC. They studied the ability of lymphocytic choriomeningitis virus (LCMV)-specific CTL's to lyse LCMV infected target cells. However, LCMV infected targets were first treated with antibodies against surface H-2K and H-2D glycoproteins. Antibody blocking of H-2 molecules on these targets significantly inhibited their lysis by syngeneic CTL's. Therefore, it appeared that CTL lysis occurred only when viral antigen was presented in the context of H-2D or H-2K on target cell surfaces. H-2D or H-2K restriction of anti-viral CTL's has been confirmed in several systems. For example, L cell targets, of the $H\text{-}2^k$ haplotype, were transformed to express $H\text{-}2K^b$ (i.e., Class I H-2K molecules of the $H\text{-}2^b$ haplotype). These transformed cells infected with influenza virus were significantly lysed by $H\text{-}2K^b$ restricted anti-influenza CTL's (Mellor et al., 1982) (Non-transformed L cells were not lysed by these effectors).

MHC restriction of anti-VSV CTL has also been demonstrated. Depending on the mouse haplotype studied, CTL's showed more dominant restriction to one or the other allele of the H-2 locus. In the $H\text{-}2^b$ system, CTL's were shown to specifically recognize VSV with a marked preference for $H\text{-}2K^b$ compatibility (Geiger et al., 1979). Studies using $H\text{-}2^d$ CTL

clones, however, showed an H-2D^d restricted response to VSV (Rosenthal et al., 1983). Hansen et al. (1981) discovered that the H-2D region was actually composed of at least four H-2D encoded gene products: D, L, M and R. In light of this complexity, Ciavarra and Forman (1982) demonstrated that anti-VSV CTL in the H-2^d system were actually restricted by the L^d molecule.

Mice of the H-2^k haplotype were unable to generate CTL responses to VSV, despite their ability to elicit anti-VSV antibody responses (Rosenthal and Zinkernagel, 1981). Studies using recombinant H-2 haplotypes showed that unresponsiveness mapped to both the H-2K^k and H-2D^k alleles. For example, VSV infected A/J (H-2K^{k}D^d) and C3H.OH (H-2K^{d}D^k) congenic mice specifically lysed VSV infected P815 (H-2^d) targets but not VSV infected L929 (H-2^k) targets (Rosenthal and Zinkernagel, 1981).

Therefore, the present studies measuring specific CTL responses toward VSV-G were attempted in responder mouse haplotypes Balb/c (H-2^d) and C57Bl/6 (H-2^b). Our experiments intended to demonstrate specific MHC-restricted CTL activity within splenocyte effector populations from either mouse strain. Thus Balb/c mice should have responded more effectively toward syngeneic B10.D2 targets, and C57Bl/6 mice toward PAK targets. However, Balb/c effectors were shown to lyse VSV infected PAK (H-2^b) targets, and C57Bl/6 effectors lysed VSV infected B10.D2 (H-2^d) targets, demonstrating a lack

of MHC restriction in these cytotoxic activities. Interestingly, non-responder CBA/J ($H\text{-}2^k$) mice also showed significant lysis of VSV infected PAK and B10.D2 targets. Thus, the measured cytotoxic activity from these mouse strains appeared to differ from that of classically defined CTL's.

D/3. Natural Cytotoxicity to VSV Infected Targets

The existence of a non-MHC restricted killing activity against B10.D2 and PAK targets infected with VSV precluded attempts to define specific cytotoxicity in these assays. As this killing was clearly unlike classical CTL activity, experiments were performed to characterize it. Our results demonstrated that this cytotoxic activity found in Balb/c, C57Bl/6 and CBA/J mice was not derived from adherent cells or Thy 1+ cells. Based on these conclusions, it seemed possible that VSV-infected target cell lysis was due to a natural killer-like activity.

Natural Killer (NK) cells have been defined as non-adherent, non-phagocytic large granular lymphocytes that, like CTL, infiltrate infected tissue during virus infection of a host (Welsh, 1978). NK cells provide natural resistance to certain viruses early in infection, whereas CTL's are generated later in infection and are responsible for virus clearance and recovery of the host (McIntyre et al., 1988). NK cells have also been shown to spontaneously lyse certain tumor and virus infected cells *in vitro* without the

requirement for MHC restriction or previous exposure to antigen (Herberman et al., 1975; Kiessling et al., 1975). Both CTL and NK cells appear to kill their targets by degranulation and insertion of protein channels into the target cell membrane (Podack et al., 1985).

Several studies have attempted to define the role of NK cells in the resistance of mice to viral infection. Bukowski et al. (1983) examined NK activity against LCMV, murine cytomegalovirus (MCMV), mouse hepatitis virus (MHV) and vaccinia virus. In these experiments, mice were depleted of NK cells by intravenous injection with rabbit anti-asialo GM_1 antiserum. Asialo GM_1 is a neutral glycosphingolipid present at high concentrations on the surface of NK cells (Kasai et al., 1980; Habu et al., 1981). Mice treated in this manner were then injected with LCMV, MHV, MCMV and vaccinia and later assayed for virus concentrations in their spleens and livers. It was discovered that antibody treated mice had reduced NK activity and higher titres of MHV, MCMV and vaccinia virus, compared to untreated control mice (Bukowski et al., 1983). Since LCMV titres were not significantly different in the two mouse populations, these investigators concluded that the relative importance of NK in resistance to virus infection may vary depending on the virus (Bukowski et al., 1983).

The possibility that natural cytotoxicity isolated from VSV and AdG12 infected mice was induced by virus infection, was refuted by studies using non-immunized Balb/c, C57Bl/6

and CBA/J mice. Splenocytes from these naive mice also demonstrated killing against VSV infected B10.D2 and PAK targets. Thus, it seemed that VSV infected target cells were more sensitive to a natural cytotoxicity inherent in these non-immunized mice.

Examples where cytotoxicity by human NK cells is augmented against virus infected human targets have been reported (Santoli et al., 1978). In the murine system, Piontek et al. (1980) showed that normal spleen cells with NK characteristics were more cytotoxic for mouse 3T3 cells infected with vaccinia or HSV-1 virus than for uninfected mouse cells. Cytotoxicity was furthermore not abrogated by removal of adherent cells, B cells or Thy 1+ cells and was present in T-cell deficient (nude) mice (Piotek et al., 1980).

A number of theories have suggested a mechanism for this augmented NK activity. It was thought that normal NK cells were induced to a greater lytic activity <u>in vitro</u> by interferon (IFN) released from virus infection 3T3 targets (Piontek et al., 1980). Santoli et al. (1978) also presented evidence in the human system that IFN enhances NK activity.

Holmes et al. (1986) reported evidence that spleen cells from non-immunized mice exhibit a high level of cytotoxicity against MHV infected target cells. This natural killer-like activity appeared to correlate with the ability of MHV virions to bind to leukocyte effectors. Also, MHV infected targets were treated with antibodies against E2, a virus specific

glycoprotein displayed on infected cell surfaces. Anti-E2 antibodies were shown to block NK activity. Data suggested that natural MHV associated cytotoxicity depended on the ability of the viral E2 protein to bind to receptors on the surface of NK-like spleen cells (Holmes et al., 1986).

Other evidence supports this notion that NK cells recognize viral glycoproteins on the surface of infected target cells (Moller et al., 1985). In this study, nylon-wool nonadherent mouse spleen cell effectors demonstrated enhanced lysis of a number of VSV infected target cells compared to uninfected cells. These effectors were isolated from non-immunized mice and were shown to display surface moieties characteristic of NK cells (Moller et al., 1985). To reveal a mechanism for enhanced NK activity against these targets, this group made use of VSV ts mutants previously used in clarifying VSV protein recognition by CTL's (Zinkernagel et al., 1978b; Hale et al., 1978). Target cells infected with ts 045 did not express VSV-G on their surfaces at non-permissive temperatures. NK cells failed to bind to or lyse these mutant targets, suggesting that VSV-G had a role in recognition by NK cells (Moller et al., 1985). Interestingly, NK cells also failed to lyse targets infected with mutants ts G31 and ts G33. Target cells infected with either of these mutants did not produce M protein at non-permissive temperatures, although surface G protein could be detected by immunofluorescence and FACS analysis (Moller et al., 1985).

In wildtype VSV, M protein serves as a matrix to associate cytoplasmic VSV nucleocapsid with the cytoplasmic domain of transmembrane G protein (Zakowsky and Wagner, 1980). It was suggested that both M and G proteins were required for lysis of VSV infected targets by NK cells. To confirm this, COS cells were transformed to express VSV-G (but not VSV-M) and, unlike VSV infected COS cells, they were not significantly lysed by NK cells (Moller et al., 1985). Thus, it was possible that VSV-G was the target for NK lysis in these studies, but only in a conformation based on its interaction with VSV-M (Moller et al., 1985). In this manner, VSV infected target cells may have been more susceptible to NK lysis.

In the context of these observations, VSV infected B10.D2 and PAK targets in the present studies should be examined for susceptibility to lysis by cloned NK cell lines. This may support the notion that cytotoxicity measured in these assays is actually due to an NK-like activity. Other experiments may include treatment of spleen cell extracts with anti-asialo GM_1 antiserum to deplete NK cells. If treated effector groups show an abrogated amount of cytotoxicity toward VSV infected targets compared to untreated effectors, this may suggest that NK cells were responsible for the detected killing activity.

Evidence to refute the existence of NK cells in these spleen cell extracts comes from the fact that NK-sensitive YAC-1 targets were not demonstrably lysed by naive or virus

primed effectors from Balb/c and C57Bl/6 mice. However, the susceptibility of our YAC-1 cells to NK killing was not tested in a positive controlled manner. YAC-1 cell lysis should have been demonstrated using an NK effector clone known to kill these targets. It is not, therefore, clear that NK cells were not present within these isolated splenocyte populations. At any rate, the fact that this cytotoxicity against VSV infected targets seemed to be non-MHC restricted, and was not derived from adherent or T cells, implies that it was due to an NK-like activity.

D/4. The Specificity of the Anti-VSV CTL Response

The molecular basis by which CTL's recognize viral antigens has been of great concern with respect to the development of anti-viral vaccines. Traditionally, CTL's have been assumed to recognize viral encoded glycoproteins expressed at high levels on the surface of infected cells in conjunction with Class I MHC glycoproteins. More recent evidence shows that CTL's can also recognize viral antigens in the context of Class II MHC (Lukacher et al., 1985; Jacobson et al., 1984). Techniques such as DNA mediated gene transfer and the construction of viral recombinants have been used to determine the specificity of CTL's in a number of viral systems. To support the idea that viral glycoproteins are the major CTL targets, Wiktor et al. (1984) constructed vaccinia recombinant viruses expressing the rabies virus

glycoprotein gene. They demonstrated that rabies virus induced CTL specifically lysed syngeneic targets infected with the vaccinia recombinant. Furthermore, by comparing CTL lysis of rabies virus infected and vaccinia recombinant infected targets, they found that a significant portion of anti-rabies virus CTL were specific for the glycoprotein. The glycoprotein was therefore considered a dominant target antigen for anti-rabies virus CTL. Similarly, in anti-herpes virus cell-mediated immunity, HSV-1 specific CTL's were thought to predominantly recognize cell surface HSV-1 glycoprotein on both human and murine HSV-1 infected targets (Glorioso et al., 1985; Zarling et al., 1986; Rosenthal et al., 1987).

Studies using temperature sensitive (ts) mutant strains of VSV implied that VSV-G was the major target antigen recognized by anti-VSV CTL (Zinkernagel et al., 1986b; Hale et al., 1978). Mouse cells infected with ts 045 did not express cell surface VSV-G at the non-permissive temperature of 39.5°C. These targets were not lysed as well as VSV infected targets (expressing intact VSV-G) by anti-VSV CTL (Zinkernagel et al., 1978b). Hale et al. (1978) supported these observations using ts M501, a VSV mutant also defective in G protein production. In targets infected with ts M501 at non-permissive temperatures, G protein does not move to the cell surface from its site of synthesis. This mutant also failed to render target cells susceptible to T cell-mediated

lysis (Hale et al., 1978). Thus, expression of VSV-G on the surface of infected target cells seemed to be necessary for lysis by VSV-specific H-2 restricted CTL.

In contrast to these original predictions, recent evidence shows that in several viral systems, internal or non-structural viral proteins are the major targets for anti-viral CTL. Influenza virus has served as a useful model for CTL specificity studies. In one report, murine L cells were transfected with genes encoding either the hemagglutinin (HA) or the nucleoprotein (NP) of influenza A. These cells were used to investigate recognition by influenza A - specific CTL (Townsend et al., 1984). It was discovered that NP transfected L cells were more effectively lysed than HA transfected targets, suggesting that NP was the major target for anti-influenza CTL. Since NP is an unglycosylated non-transmembrane protein, further studies focussed on defining a mechanism for CTL recognition of internal protein epitopes. Townsend's group demonstrated that anti-influenza A CTL's recognized short 15 amino acid length fragments of NP. Influenza NP target epitopes were defined by using cells transfected with truncated NP genes (Townsend et al., 1985) or by using synthetic NP peptides (Townsend et al., 1986b). In both cases, fragments of NP sensitized cellular immune responses better than the entire NP protein. Based on these studies, Townsend et al. (1986b) suggested that the internal NP protein was first degraded and then displayed on target

cell surfaces to anti-NP CTL. This idea was consistent with studies where inhibitors of internal protein processing prevented effective antigen presentation to Class II restricted T cells (reviewed by Unanue, 1984). For example, the lysosomotropic drug chloroquine was shown to inhibit antigen processing necessary for presentation of influenza NP and HA to Class II restricted human T cells (Fleischer et al., 1985).

To confirm the notion that anti-influenza CTL recognized internal proteins Townsend et al. (1986a) constructed recombinant vaccinia virus containing coding regions for the transmembrane HA protein. However, they deleted the sequence encoding the N terminal signal peptide so that HA was not expressed on the surface of infected targets. In these experiments, HA specific CTL lysed targets expressing leaderless intracellular HA as efficiently as targets expressing intact surface HA (Townsend et al., 1986a). Thus, CTL's were able to recognize epitopes on the HA protein whether it was displayed on the cell surface or internally. Internal or non-structural virus proteins were discovered to be major CTL targets in other viral systems such as respiratory syncytial virus (Bagham et al., 1986) and HSV-1 (Martin et al., 1988), consistent with these observations in the influenza virus system.

Based on these discoveries, it was necessary to re-evaluate studies on anti-VSV CTL target specificity.

Rosenthal and Zinkernagel (1980) isolated a population of anti-VSV CTL's that cross-reactively recognized two serotypes of VSV (Indiana and New Jersey). CTL's raised against one serotype demonstrably lysed targets infected with the other serotype. As mentioned, VSV-G was considered the major target for anti-VSV CTL (Zinkernagel et al., 1978b). Since antibodies against VSV-G defined the VSV serotypes (Lefrancois and Lyles, 1982), it was suggested that CTL's recognized cross-reactive epitopes on VSV-G (Lefrancois and Lyles 1983b). However, several monoclonal antibodies bound to different G protein epitopes on the surface of infected target cells were unable to block anti-VSV CTL reactivity (Lefrancois and Lyles, 1983b). It was therefore suggested that other VSV proteins served as CTL targets.

Puddington et al. (1986) investigated this hypothesis by generating cloned target cell lines constituitively expressing VSV-G or VSV-N. In these experiments, anti-VSV CTL's were produced <u>in vitro</u> by coculturing spleen cells from mice VSV primed four weeks previously with spleen cells irradiated and VSV infected. These irradiated spleen cells were thought to secondarily stimulate VSV memory CTL's. Secondary anti-VSV CTL lysed VSV-N transfected targets to a greater extent than VSV-G targets. Furthermore, cold target competition assays showed that VSV-G expressing targets were unable to inhibit any of the anti-VSV CTL (Puddington et al., 1986), suggesting that N was the major target for secondary anti-VSV CTL.

Experiments using vaccinia recombinant virus vectors containing VSV genes lent further support to these conclusions. Vectors were constructed that contained either the VSV-N or VSV-G coding region (Yewdell et al., 1986). Once again, targets infected with the vaccinia-VSV-N virus were killed more effectively by anti-VSV CTL than vaccinia-VSV-G infected targets. Additionally, it was discovered that most N-specific CTL's were also cross-reactive, i.e., they specifically lysed targets infected with different VSV serotypes to similar degrees (Yewdell et al., 1986). By secondary in vitro stimulation methods, similar to Puddington et al. (1986), they isolated a population of serotype-specific CTL's and unlike the cross-reactive populations, these CTL's were shown to effectively lyse targets infected with vaccinia-VSV-G. Thus, it was possible that although most anti-VSV CTL recognize the N protein, VSV-G may be a target for a small population of serotype-specific CTL (Yewdell et al., 1986). These authors postulated that earlier reports using VSV ts mutants defined VSV-G as the major CTL target because they only raised primary anti-VSV CTL's. Zinkernagel et al. (1978b) showed that primary anti-VSV CTL populations are often predominantly serotype specific. In these more recent studies, secondary anti-VSV CTL's were shown to be largely cross-reactive and, consistent with observations in other viral systems, they recognized internal VSV-N as a dominant target antigen (Puddington et al., 1986; Yewdell et al.,

1986).

D/5. The AdG12 Recombinant Virus Model and Its Implications

AdG12 was chosen as a model to study the use of adenovirus vectors to elicit specific CTL responses in mice. However, in light of recent evidence on anti-VSV specificity, AdG12 would only serve to measure responses against a minor CTL target antigen. A more useful model therefore would involve the construction of adenovirus recombinants containing the coding regions for VSV nucleoprotein (N). Ad recombinant-N virus infection of mice may certainly induce a larger anti-VSV CTL response, since N is now considered the major CTL target antigen (Puddington et al., 1986; Yewdell et al., 1986). For this reason, it would also be more beneficial to study CTL responses against targets specifically expressing VSV-N.

The present studies also fall short of providing a significant model for anti-VSV cell-mediated immunity since only primary CTL assays were performed. Yewdell et al. (1986) demonstrated that when spleen effectors from VSV primed mice are re-stimulated in vitro with irradiated infected splenocytes (secondary stimulation), a population of N-specific, serotype cross-reacting CTL's are obtained. In our studies, cell-mediated immunity should be examined using CTL populations that respond effectively toward several VSV serotypes. This would certainly be beneficial toward the

development of recombinant virus vaccines against VSV.

Secondary stimulation of spleen effectors in vitro was attempted but unfortunately was unsuccessful. Spleen cells were removed from mice primed 3 weeks previously with AdG12 or VSV according to earlier studies (Zinkernagel et al., 1978b). These splenocytes were cocultured for several days with irradiated peritoneal exudate cells (PEC's) infected with UV-inactivated VSV or AdG12. Infected PEC's were thought to act as antigen presenting cells that would stimulate VSV-specific CTL to proliferate in response to presented viral antigens. However, in these attempts, CTL's did not proliferate by specific viral antigen stimulation. In fact, spleen cells from AdG12 and VSV primed mice seemed to proliferate when cultured in vitro with uninfected PEC's (data not shown). Thus, it was not possible to isolate a population of secondarily stimulated CTL's against VSV. It was not clear why these attempts were unsuccessful but some changes in methodology may prove to be beneficial. For example, in other anti-VSV CTL studies, secondary in vitro stimulation was performed using irradiated antologous spleen cells (Yewdell et al., 1986). These spleen cells may serve as better presenter cells than PEC's when infected with VSV or a recombinant virus. Also, anti-VSV secondary assays performed by Puddington et al. (1986) involved restimulation with live rather than UV-inactivated VSV. Future attempts to propagate secondary anti-VSV CTL should be considered in light of these

methodological differences.

The potential for Ad recombinant vectors to elicit anti-viral immune responses have been studied in a number of virus models. For example, Morin et al. (1987) constructed Ad vectors carrying the hepatitis B virus surface antigen coding sequence. Using a hamster model, they demonstrated that this vector could induce an antibody response against the hepatitis B surface antigen. Recombinant AdG12, as mentioned, has been recently shown to induce the production of neutralizing antibodies to VSV in a variety of animal hosts including mice (Prevec et al., 1989). A successful model using Ad recombinant viruses to induce cell-mediated anti-VSV responses in mice would therefore supplement these studies.

D/6. Summary and Conclusions

The present experiments have attempted to analyse the use of adenovirus recombinant AdG12 to induce specific cell-mediated immune responses against VSV in mice. Using a standard ^{51}Cr release assay, the response of VSV and AdG12 primed spleen cell effectors was measured against labelled syngeneic target cells.

Several murine cell lines were examined for their ability to serve as targets in these assays. Of the cell lines tested, H-2^d B10.D2 and H-2^b PAK cells demonstrated productive infection with AdG12 and subsequent expression of VSV-G by 36 hours post-infection. Therefore, these cell lines were used

as syngeneic targets to measure primary cell-mediated immunity in Balb/c and C57Bl/6 mice.

The kinetics of CTL activity in mice intravenously infected with AdG12 were measured in primary assays against infected targets. It appeared that mice primed with AdG12 demonstrated peaked cytotoxic activity six days post-infection against syngeneic targets infected with Ad5 wt or AdG12. This activity was considered to be that of Ad protein-specific CTL's.

In an attempt to examine the specificity and MHC restriction of AdG12 primed effectors, a non-MHC restricted cytotoxicity against VSV infected targets became apparent. In fact, splenocyte effectors isolated from immunized and non-immunized mice showed significant lysis of VSV infected B10.D2 and PAK targets. These effectors were isolated from Balb/c, C57Bl/6 and CBA/J mice.

It was necessary to characterize this inherent cytotoxic activity. Depletion experiments revealed that the killing activity was not due to adherent cells or T cells within splenocyte populations. This activity therefore appeared to be that of an NK-like effector. The presence of NK-like cytotoxicity precluded subsequent efforts to define the specificity of anti-VSV responses in these mice.

Based on recent evidence (Yewdell et al., 1986), it appears that secondary <u>in vitro</u> stimulation of splenocytes from virus primed mice would be more beneficial in obtaining

anti-VSV CTL. Furthermore, the construction of an Ad recombinant vector containing the coding sequence for VSV-N would provide a more useful model to study the cell-mediated immune response toward VSV.

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